Persisting virulence of *T. pallidum* after incubation with penicillin in Nelson-Mayer medium

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Several investigators have shown, in syphilitic infections of both man and animals, that after prolonged and intensive penicillin therapy some viable organisms can still be found in certain body tissues and fluids, notably the lymph nodes, cerebrospinal fluid, and aqueous humour (Collart, Borel, and Durel, 1962; Collart and Durel, 1964; Del Carpio, 1963; Yobs, Olansky, Rockwell, and Clark, 1965; Yobs, Clark, Mothershed, Bullard, and Artley, 1968; Rimbaud and Roux, 1965; Smith, Singer, Reynolds, Moore, Yobs, and Clark, 1965; Ehrmann, 1967; Smith, 1969; Nicolau, Badanoiu, Nicolau, and Gavrilscou, 1969; Rice, Dunlop, Jones, Hare, King, Rodin, Mushin, and Wilkinson, 1970).

Although the validity of these findings has sometimes been doubted, the phenomenon has become less debatable with the increasing number of investigators who have observed it. The search for an explanation has produced several hypotheses. The possibility of re-infection was effectively ruled out in animal experiments. It is also unlikely that these treponemes were not all *Treponema pallidum*.

Two further hypotheses may be explored:

(1) That the penicillin may not reach adequate antibacterial levels in some of the body tissues or fluids. Recent investigation has shown that this is highly unlikely. Pechère, Franceschini, and Collart (1971), using the *Sarcina lutea* plate method (Bennett, Brodie, Benner, and Kirby, 1966), have measured penicillin concentrations in the serum, lymph nodes, and testes of syphilitic and normal rabbits, and have shown that tissue concentrations, though lower than those of serum, are none the less adequate for treponemicidal effect at the doses customarily given. Penicillin concentrations in infected testes were higher than in normal testes.

(2) That certain factors inhibit the antibacterial activity of penicillin, even when in contact with

Treponema pallidum. This apparent resistance to high concentrations of penicillin has been demonstrated *in vitro* and will be discussed below.

Material and methods

GENERAL PRINCIPLES

Penicillin G was added to suspensions of two different strains of *Treponema pallidum* to give concentrations of from 0.005 to 50,000 units/ml. After the suspensions had been incubated for 15, 48, or 72 hours, the penicillin was neutralized by the addition of penicillinase. Portions of the suspensions were then inoculated into the testes of a fresh rabbit to determine the virulence of the treponemes (Figure).

STRAINS

Two strains were used:

- (1) Unmodified virulent Nichols strain (Nichols, 1914).
- (2) A Nichols strain modified by repeated inoculations into rabbits which had been given increasing doses of penicillin G. The date of the first passage was September 21, 1966, and the total number of passages was 200. The last treatment was 15,000 units/kg./day Bipenicillin for 5 days.

Incubation of Treponema pallidum with penicillin

Treponemes were taken from infected testes and counted under a darkfield microscope; they were then diluted in Nelson-Mayer medium to give a final concentration of 10^7 organisms per ml. (Magnuson, Eagle, and Fleischman, 1948). Penicillin was also diluted in Nelson-Mayer medium and the desired concentrations were added to the treponeme suspensions (Table). The organisms were then incubated with the antibiotic at 35°C. in an atmosphere of 95 per cent. nitrogen and 5 per cent. CO₂. After periods of 15, 48, or 72 hours, the penicillin was neutralized by the addition of appropriate amounts of penicillinase. The organisms were then examined by darkfield microscopy and injected into the testes of a fresh rabbit. The rabbits were observed for the possible development of syphilitic orchitis. When orchitis developed, its nature was checked by examination under the darkfield microscope. Each experiment was controlled by a parallel experiment using treponeme suspensions without penicillin.

Altogether 66 rabbits of an unselected breed, weighing from 2.5 to 3 kg., were used in this investigation. They were given natural feed without antibiotics.

Results

These are summarized in the Table.

Discussion

These results appear to be contradictory. Whereas the antibacterial activity of penicillin has been amply demonstrated *in vivo*, these studies *in vitro* indicate that *Treponema pallidum* remained virulent after incubation with high concentrations of penicillin.

Before attempting to interpret these results, certain questions must be answered:

(1) The possible inactivation of the penicillin during the incubation period. Assays performed after 48 hours' incubation showed a decrease in activity of only 10 to 15 per cent., so that inactivation of the antibiotic is ruled out as a possible explanation.

(2) The relationship between the motility and viability of *Treponema pallidum*. Frequently, just before the intratesticular inoculation after incubation with penicillin, the treponemes appeared to be immobile, and had also lost some of their spiral structure. But that some at least of them remained viable is proved by their ability to produce orchitis. This suggests that morphological abnormalities and loss of motility do not necessarily correspond with loss of viability. Moreover, it is difficult to distinguish between reduced motility and that due to Brownian movement. Thus the degree of motility of the organisms cannot be used as the only criterion by which to judge the activity of an antibiotic *in vitro*.

Although the survival of *Treponema pallidum* in media containing high concentrations of penicillin is somewhat surprising, there is an obvious explanation for this apparent resistance to penicillin G. Treponemes are known to remain viable in Nelson-Mayer medium without multiplying. From original studies on penicillin (Fleming, 1929; Abraham, Gardner, Chain, Heatley, Fletcher, Jennings, and Florey, 1941), this antibiotic is known to act only on growing organisms, so that the quiescence of the treponemes should permit survival even in high concentrations of the antibiotic. Thus, the phenomenon is probably not due to a genuine resistance to penicillin on the part of the treponemes. Furthermore, the orchitis produced in the rabbit by intratesticular inoculation of these survivors *in vitro* is curable by

TABLE Results of intratesticular inoculations into rabbits of *Treponema pallidum* after incubation with penicillin in Nelson-Mayer medium

Concentrations of benzyl-penicillin (units/ml.)	Results of inoculations					
	15 hrs*		48 hrs*		72 hrs*	
	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2
0 (Controls)	+ (4/4)‡	+ (5/5)	+ (1/1)	+ (1/1)	-(0/2)	-(0/2)
0.005 to 500	+ (10/10)	+ (10/10)	+ (5/5)	+ (5/5)	-(0/4)	-(0/4)
1,000 to 10,000	+ (3/3)	+ (3/3)	-(2/2)	-(2/2)	-(0/2)	-(0/2)
Above 10,000	-(0/2)	-(0/2)				

+ = orchitis - = no orchitis ‡ (n/m) = $\frac{\text{number of positive responses (orchitis)}}{\text{number of tested rabbits}}$

* = period of incubation

Strain 1 = Nichols Strain 2 = modified Nichols

normal doses of penicillin. The *Treponema pallida* in the special conditions of these experiments are 'indifferent' to penicillin, rather than 'resistant', and this only because they have been placed in conditions that are adverse to growth.

A parallel may be drawn between this study *in vitro* and observations made in both man and animals of the failure of prolonged and intensive penicillin therapy to cure certain syphilitic infections. *Treponema pallida* are known to persist for months and years in body tissues with little or no growth. This quiescence would permit the organisms to be 'indifferent' to penicillin treatment just as the treponemes survived incubation with penicillin *in vitro* in the present study.

Summary

Treponema pallidum organisms (Nichols strain) were incubated in Nelson-Mayer medium together with concentrations of benzyl penicillin from 0.005 to 50,000 units/ml., and 15, 48, and 72 hours later the treponemes were inoculated intratesticularly into healthy rabbits. Despite 15 hrs' contact with penicillin 10,000 units/ml. or 48 hrs' contact with penicillin 500 units/ml., the treponemes retained their ability to cause orchitis. This finding is attributed to the fact that treponemes were not dividing and were therefore 'indifferent', rather than resistant, to penicillin.

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Persistence de la virulence de *T. pallidum* après incubation dans le milieu de Nelson-Mayer, en présence de pénicilline

SOMMAIRE

Des *Treponema pallida* (souche Nichols) furent incubés dans du milieu de Nelson-Mayer contenant de la benzyl-pénicilline aux concentrations de 0,005 à 50.000 unités/ml., et 15, 48, et 72 heures après, ces tréponèmes furent inoculés par voie testiculaire à des lapins sains. Malgré 15 heures de contact avec 10.000 unités/ml. ou 48 heures de contact avec 500 unités de pénicilline par ml., les tréponèmes avaient conservé la faculté de créer des orchites. Ce résultat est attribué au fait que les tréponèmes n'étaient pas en phase de division et étaient, vis-à-vis de la pénicilline, 'indifférents' plutôt que résistants.