INDUCTION OF L-FORMS OF *HAEMOPHILUS INFLUENZAE* IN VITRO

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PLATE XX

HAEMOPHILUS INFLUENZAE is the commonest cause of persistent or recurrent bacterial infection in chronic bronchitis. Indeed, the major problem in the chemotherapy of the condition is the eradication of this organism from the respiratory tract of patients with advanced disease; all too frequently, apparently successful treatment is followed rapidly by relapse of infection caused by bacteria that have evidently been only temporarily suppressed (May, 1972).

Ampicillin has been used more extensively than any other bactericidal drug in attempts to eradicate *H. influenzae* in these patients, but the results have been generally disappointing. Occasionally, high dosage (1 g 6-hourly) for a few days is followed by prolonged absence of pus from the sputum, apparently because the haemophilus has been totally cleared from the respiratory tract, but such a response is uncommon. May and Delves (1965) suggested that the frequent failure of ampicillin to eradicate the haemophilus could be attributed to the relatively low concentrations attainable in the respiratory secretions. However, Lapinski and Flakas (1967) proposed an alternative explanation. They suggested that L-forms of the organism are induced by the ampicillin and, being resistant to penicillins, persist throughout treatment. When treatment is stopped they are presumed to revert to the vegetative form of the organism and to re-invade the bronchial mucosa. It has generally been supposed that the osmotically labile L-forms would not survive in the environment to which they would usually be exposed, but work carried out in this Department indicates that the L-forms of *H. influenzae* withstand a wide range of osmolality (Roberts *et al.*, 1974).

We now describe some observations on the induction of L-forms of *H. influenzae*, mainly by penicillins, on nutrient agar *in vitro* and preliminary observations on the behaviour of L-forms in the environment provided by sputum.

In this paper, the term "L-forms" is used in its widest sense to denote morphological variants derived from typical bacteria by the action of inducing substances, and carries no implication concerning the degree of cell-wall defect of the organisms.

MATERIAL AND METHODS

Bacterial strains

The seven strains of *H. influenzae* used in these studies were non-capsulated and had been isolated from the sputum of patients attending the Brompton Hospital.

Culture media

XV broth and XV agar. These were made from meat-digest broth (Baker and Breach, 1967), the latter with the addition of Oxoid Agar no. 1, 1 g per 100 ml, plus X factor (haemin, BDH) and V factor (nicotinamide adenine dinucleotide, BDH) at final concentrations respectively of 3 and 0.3 mg per 100 ml.

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Sputum agar. This was prepared from mucoid sputum obtained from patients with chronic bronchitis. Samples were homogenised by ultrasonic vibration by means of a "Soniprobe 1130" (Dawe Instruments Limited, Acton, London), with an output current of 3-4 A for 20-30 s. To the homogenate was added an equal volume of 3% agar in distilled water, previously melted and cooled to 50°C. The mixture was poured into 9-cm petri dishes, to a minimal depth, since its opacity seriously impaired the efficiency of phase-contrast examination of organisms on its surface. The sputum was not sterilised before being made into medium; provided that it did not contain pseudomonads or enterobacteria, no problems were encountered from overgrowth of or interference with the test organisms by other bacteria.

Preparation and observation of cultures

On XV agar. XV-agar plates approximately 3-mm thick were flooded with 3-h XV-broth cultures of the test strains. The excess was removed and the plates were dried at 37°C. Blocks, 6 mm in diameter, were cut out with a sterilised cork-borer and placed in 9 cm petri dishes containing 5 ml of XV broth, to which inducing agents or other substances were added as required. Several blocks were prepared for each experiment, some for direct examination and some for subsequent subculture to determine the viability of the organisms (see below). The preparations were incubated at 37°C and examined by phase-contrast microscopy at appropriate time intervals. To avoid fogging of the microscope objective (magnification $\times 40$), glass coverslips were applied to the agar blocks for examination. However, because the continued presence of a coverslip caused premature disintegration of the L-forms after 24 h, preparations for examination later than the day on which an experiment was set up were separated from the rest and coverslips applied immediately before examination. For preparations up to 6-h-old the coverslips were left in position throughout the observation period.

In some experiments, the viability of the organisms was monitored at intervals by subculture on XV agar containing no L-form-inducing agents. At the relevant time, a block was applied to the XV agar, inoculated surface downwards, and pushed across its surface; viable L-forms gave rise, after overnight incubation at 37°C, to colonies of normal *H. influenzae*.

Removal of the inducing agent from a preparation was achieved by pipetting off the broth surrounding the block and replacing it with XV broth containing no inducer. This process was repeated 15 min. and 30 min. later. In experiments in which penicillin, 1 μg per ml, was used as the inducer, assay of the "washings" showed no detectable penicillin (less than 0.01 μg per ml) in the second washing.

On sputum agar. Because of difficulties in obtaining suitable sputum samples of sufficient size, observations were limited to the induction and survival of L-forms during continuous exposure to inducing agents on sputum-agar plates.

The plates were inoculated with the test organism as described above. The inducing agent was introduced either as a solution placed in a hole cut in the medium with a 6-mm cork-borer immediately after inoculation or it was incorporated at a known concentration in the medium before the plate was poured. Viability of organisms was determined by transfer of blocks to XV agar plates as described above.

RESULTS

Induction of L-forms by penicillin

When *H. influenzae* strain 909 (see fig. 1) was exposed to penicillin, 1 μg per ml, the following stages were observed.

Stage I. During the first 3 h, the organisms elongated to form threads which eventually reached about 30 μ in length (fig. 2).

II. In the next 2 h, one or more dense granules appeared in the threads (fig. 3).

III. During the next 1-1½ h, the granules began to enlarge and the threads to shorten (fig. 4).

IV. After 24 h, the threads had almost disappeared and the granules had developed into round bodies about 5 μ in diameter (fig. 5).

V. After 48 h, the round bodies had become larger and vacuolated and started to disintegrate (fig. 6).

Although in the first few hours of each experiment some overlap between the stages was observed at any given time, this was usually of minor degree, and the stages could be defined with considerable precision. The formation of L-forms was depicted graphically by plotting the stages of development against time, as exemplified in fig. 7.

Fig. 7 also shows the effect of varying the concentration of penicillin. No morphological changes were observed in organisms exposed to less than $1.0 \mu\text{g}$ of penicillin per ml, which was found to be the MIC of this antibiotic for the strain. At concentrations of penicillin between $1 \mu\text{g}$ and $100 \mu\text{g}$ per ml, L-forms developed at an increased rate and round bodies tended to develop directly from bacterial forms, and at $100 \mu\text{g}$ per ml, stage IV was reached in only 3 h. No further increase in the rate of development of L-forms was brought about by increasing the penicillin concentration to $1000 \mu\text{g}$ per ml.

Typical *H. influenzae* was recovered from each preparation exposed to penicillin, $10 \mu\text{g}$ or less per ml for up to 48 h. No viable organisms were recovered from the preparations exposed to $100 \mu\text{g}$ per ml after 2 h.

Induction by penicillin of L-forms of six other strains of *H. influenzae* was essentially similar, but the rate of development varied somewhat from strain to strain.

It was found that L-forms of strain 909 developed through the same stages and at the same rate after exposure of the organisms to penicillin $1 \mu\text{g}$ per ml for only $2\frac{1}{2}$ h and subsequent incubation in the absence of antibiotic.

Effect of other inducers

Amoxycillin. As with penicillin, the MIC of amoxycillin was also the minimal L-form-inducing concentration for each of the seven strains of *H. influenzae* tested. With strain 909 and one other strain, the rate of induction by amoxycillin at the MIC and at $10 \mu\text{g}$ per ml was substantially slower than that by penicillin at similar concentrations. Of the remaining

FIG. 7.—Development of L-forms of *Haemophilus influenzae* when exposed to penicillin: — = $0.1 \mu\text{g}$ per ml; - - - = $1.0 \mu\text{g}$ per ml; — · — = $10.0 \mu\text{g}$ per ml; · · · = 100.0 and $1000.0 \mu\text{g}$ per ml. — = Control.

strains, one showed more rapid induction by amoxycillin than by penicillin, while four were induced at the same rate by the two antibiotics.

Glycine. This substance is known to induce L-forms in a number of bacterial species, and is a constituent of sputum. The minimal effective concentration of glycine for the production of L-forms by strain 909 was found to be 7 mg per ml, which is very considerably higher than the concentration in sputum (about 7.5 μ g per ml). The stages of L-form induction were similar to those observed with induction by penicillin with the exception that thread forms were much less prominent.

Combinations of penicillin and glycine. The table shows that, when strain 909 was exposed to mixtures of penicillin and glycine, some reduction in the minimal inducing concentration of each substance was observed.

Induction and survival of L-forms on sputum agar

On sputum agar inoculated with *H. influenzae* strain 909, and with a hole filled with an aqueous solution of penicillin 1000 μ g per ml, numerous clusters of round bodies morphologically similar to stage-V L-forms developed (fig. 8). On transfer to XV agar, normal haemophilus colonies developed after overnight incubation. Strain 5051 showed a similar development of round bodies, and a similar degree of survival, when grown on sputum agar containing amoxycillin 0.3 μ g per ml and on XV agar containing this antibiotic 0.6 μ g per ml.

DISCUSSION

We have shown that L-forms of *H. influenzae* are induced by penicillin or amoxycillin at their respective MICs, and survive for at least 48 h, not only on a nutrient-agar medium but also on an agar medium in which the mucoid sputum of a patient with chronic bronchitis provides the only nutrient and to which no osmotic stabiliser is added. These L-forms revert to the vegetative state when subcultured on to antibiotic-free medium.

Sputum normally contains L-form inducing substances, such as glycine, lysozyme, and often antibody, and, although their usual concentrations are probably less than those necessary for induction, their presence may possibly lower the concentration of antibiotic required to induce L-forms. Evidence for an additive effect was obtained from experiments in which sub-inducing concentrations of glycine lowered the minimal inducing concentration of penicillin, although the concentration of glycine required to produce this effect was much higher than that in sputum. The minimal inducing concentration of amoxycillin for *H. influenzae* (strain 5051) on sputum agar was 0.3 μ g per ml, a concentration that might be

TABLE
*Effect of the combination of various concentrations of penicillin and glycine
on induction of L-forms of Haemophilus influenzae (strain 909)*

Glycine (mg per ml)	L-form induction*, in the presence of glycine at the stated concentration, and of penicillin (μ g per ml)							
	1.0	0.5	0.25	0.12	0.06	0.03	0.01	0.0
7.0	+	+	+	+	+	+	+	+
5.0	+	+	+	+	+	+	—	—
2.5	+	+	+	—	—	—	—	—
1.2	+	—	—	—	—	—	—	—
0.6	+	—	—	—	—	—	—	—
0.0	+	—	—	—	—	—	—	—

* + = Stage IV of L-form induction (see text) reached after incubation for 24 h; — = stage IV of L-form induction not reached after incubation for 24 h.

L-FORMS OF *HAEMOPHILUS INFLUENZAE* FIG. 1.—Normal forms of *Haemophilus influenzae*.

FIG. 2.—Stage-I L-forms, 3 h; elongation to form threads.

FIG. 3.—Stage-II L-forms, 5 h; dense granules in threads.

FIG. 4.—Stage-III L-forms, 6 h; granules enlarged, threads shortened.

FIG. 5.—Stage-IV L-forms, 24 h; threads almost disappeared, granules developing into round bodies.

FIG. 6.—Stage-V L-forms, 48 h; round bodies larger, vacuolated and disintegrating.

FIG. 8.—Stage-V L-forms on sputum agar.

attained in patients undergoing treatment with this antibiotic (May and Ingold, 1972). The relatively short exposure required (2½ h for penicillin) suggests that the sputum concentration following a single dose might be sufficient to induce L-forms. If L-forms do survive in the respiratory secretions, it may be that *H. influenzae* cannot be eradicated from the respiratory tract by penicillins, no matter how high the local concentration may be—a conclusion contrary to the belief expressed previously by May and Delves (1965).

SUMMARY

The induction of L-forms of *Haemophilus influenzae* by penicillin, amoxycillin and glycine has been studied *in vitro* on a nutrient-agar medium. The minimal inducing concentrations of the antibiotics were generally the same as their minimal inhibitory concentrations, but the addition of a sub-inducing concentration of glycine lowered the minimal inducing concentration of penicillin.

Preliminary observations have shown that L-forms are induced by penicillin or amoxycillin on a medium in which mucoid sputum forms the sole source of nutrients, and that they remain viable for at least 48 h in the absence of added osmotic stabiliser. The minimal inducing concentration on "sputum agar" is within the range of concentrations measured in sputum from patients receiving amoxycillin therapy.

The implications of these observations in relation to bactericidal therapy of haemophilus infections of the respiratory tract are discussed.

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