

PAPERS AND SHORT REPORTS

Isolation of spheroplastic forms of *Haemophilus influenzae* from sputum in conventionally treated chronic bronchial sepsis using selective medium supplemented with N-acetyl-D-glucosamine: possible reservoir for re-emergence of infection

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Abstract

The isolation rate of *Haemophilus influenzae* from patients with persistent production of purulent sputum has been increased by the routine use of a selective medium. Nevertheless, some purulent sputum still fails to yield a pathogen. The selective medium was supplemented with N-acetyl-D-glucosamine to encourage primary isolation of colony forming, spheroplastic *H influenzae*, which reverted to normal forms on sub-culture.

On the basis of in vitro experiments it is postulated that these spheroplastic forms of *H influenzae* may be induced by inadequate antimicrobial chemotherapy and may be responsible for re-emergence of symptoms in these patients during or shortly after stopping chemotherapy.

Introduction

The aim of the physician administering antibiotic to a patient with respiratory infection is elimination of the offending pathogen(s). This may be difficult to achieve in severe chronic bronchial sepsis (reviewed by Cole¹) for one or more of several reasons—for example, failure to use appropriate bacteriological culture methods² to assess microbial colonisation reproducibly

(and failure to agree on what are criteria for a pathogen in this condition); insufficient antimicrobial agent reaching the site of infection,^{3,4} and “persisters” arising in a population of bacteria as a result⁵; presence of antibiotic destroying enzyme activity in the secretions, reducing local bioavailability⁶; and deficiency of one or more host defence mechanisms.⁷

The concentration of antibiotic in bronchial secretions may be increased by raising the dose above that conventionally used,⁸ penicillin destroying enzymes may be inhibited by β -lactamase inhibitors such as clavulanic acid,⁶ and a high index of suspicion of deficiencies of immunological and non-immunological respiratory defence mechanisms will permit detection and treatment of some of these—for example, hypogammaglobulin-aemia. Mechanisms of bacterial persistence, however, are not fully understood. The late Professor Robert May hypothesised that in the case of penicillins some bacteria may escape destruction by assuming an L form, reverting to their normal morphology when the antibiotic is removed.⁵

Routine use of a selective technique⁹ for investigating patients with chronic sputum production has increased the isolation of *Haemophilus influenzae*, particularly when overgrowth of conventional cultures with pseudomonas organisms is a problem. Some sputum, however, even if cultured for prolonged periods,¹⁰ fails to yield a pathogen despite being purulent (and not composed of eosinophils). The presence of cell wall defective forms of *H influenzae* was therefore considered and a method to detect these devised.

Patients, materials, and methods

The sputum specimens were received from patients suffering from documented bronchiectasis (caused by various disorders, including postinfective damage, immunity deficiency (hypogammaglobulin-aemia), primary ciliary dyskinesia, cystic fibrosis, and Young's syndrome (obstructive azoospermia, bronchiectasis, and sinusitis)) and from patients (mainly non-smokers or ex-smokers) persistently coughing purulent sputum but in whom no bronchiectasis was detect-

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able. Most had received prolonged courses of antimicrobial agents, none of which had resulted in remission of symptoms for longer than a few weeks.

Selective culture of *H influenzae* from sputum—The method described originally⁹ was modified. Blood agar base was prepared in small batches (sufficient for six plates) to minimise damage to heat labile constituents from the melting of medium which occurs during preparation in bulk. Four grams Oxoid agar No 2 was heated with 100 ml distilled water to 121°C for 15 minutes in a pressure cooker, then cooled to 56°C. Haemin (BDH) and bacitracin (Mast Laboratories) were added to final concentrations of 3.3 and 30 mg/l respectively. The plates were surface dried and sputum (homogenised in an equal volume of phosphate buffered saline, pH 7.3, by vortex mixing) plated out in the usual manner. A disc containing factor "V" (a coenzyme essential for growth of *Haemophilus* spp) was placed on the surface of the plate, which was incubated anaerobically (to inhibit *Pseudomonas* and subdue *Branhamella* spp) for 18 hours at 37°C in an Oxoid jar containing a sachet of gas generating kit (Oxoid) which produces approximately 1800 ml hydrogen and 350 ml carbon dioxide.

Isolation of spheroplasts of *H influenzae* in primary culture—The cell walls of bacteria contain a mucopeptide (glycopeptide) as a strengthening substance. The mucopeptide is composed of *N*-acetyl muramic acid and *N*-acetyl-D-glucosamine molecules linked alternately in a chain, the former carrying peptide side chains cross linked by pentapeptide bridges. The bacterial cell wall, and particularly the mucopeptide component, is a target for various antimicrobial agents. In view of the experimental induction of L forms of *H influenzae* in vitro using penicillin,¹¹ attempts to isolate these forms were made by adding *N*-acetyl muramic acid and *N*-acetyl-D-glucosamine to the selective medium described above for culture of *H influenzae* from sputum. Homogenisation of sputum using pancreatin and ultrasonication was avoided to minimise damage to cell wall defective bacterial forms.¹² An osmotic stabiliser was not employed, as *H influenzae* have been shown to be osmotically stable.¹³

Light microscopy—Colonies of bacteria surrounding the factor "V" disc were emulsified in phosphate buffered saline on a microscope slide and a coverslip preparation examined by phase contrast microscopy using a $\times 40$ objective. Phosphate buffered saline did not lyse these spheroplasts.

Transmission electron microscopy—The organisms were scraped off agar plates and fixed in suspension in cacodylate buffered 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide. After rinsing they were embedded in a drop of 2% liquid agar and gently centrifuged. The agar was allowed to set then processed through to embedding in Araldite as routine for tissues. Semithin sections were cut and stained in 1% toluidine blue for light microscopy and suitable areas selected and trimmed for ultrathin sectioning. These were stained in uranyl acetate and lead citrate for transmission electron microscopy.

Spheroplast induction in vitro during sensitivity titration of *H influenzae* to antibiotic—A standard tube turbidity titration of the minimal inhibitory concentration of amoxycillin for *H influenzae* was carried out and the contents of all tubes examined for spheroplastic forms by light microscopy and by subculture on to solid selective medium containing *N*-acetyl-D-glucosamine.

Results

Use of supplemented selective medium in chronic bronchial sepsis—*N*-acetyl-D-glucosamine in a final concentration of 1 g/l promoted the growth of spheroplasts of *H influenzae*, whereas *N*-acetyl muramic acid did not. Of 123 recent isolates of *H influenzae*, 114 grew on media with and without *N*-acetyl-D-glucosamine. Eight of these 114 were spheroplastic on light microscopy, and this spheroplastic

form was seen only in colonies isolated on medium containing *N*-acetyl-D-glucosamine—on ordinary medium the eight isolates were composed of organisms which were morphologically normal. Nine grew only on medium containing *N*-acetyl-D-glucosamine: five of these were morphologically normal but four were found to be spheroplastic on light microscopy (fig 1), and on subculture these reverted to conventional *H influenzae*. Better growth of normal *H influenzae* was also observed when using medium supplemented with *N*-acetyl-D-glucosamine.

In vitro induction of spheroplastic *H influenzae*—During tube dilution sensitivity testing of *H influenzae* in relation to the antibiotic amoxycillin most of the spheroplasts were found in tubes ranging from 0.4 mg to 1.56 mg amoxycillin per l (table). The minimal

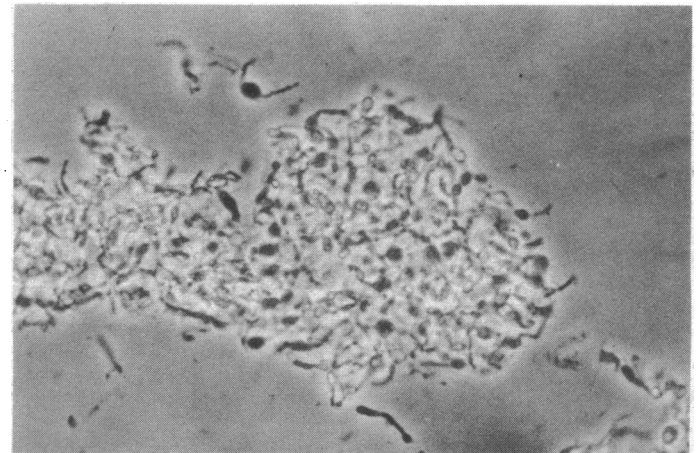


FIG 1—Wet preparation from colony of cell wall defective *H influenzae* isolated on medium containing *N*-acetyl-D-glucosamine. Phase contrast $\times 740$ (original magnification).



FIG 2—Transmission electronmicrograph of spheroplast of *H influenzae* taken from colony isolated on *N*-acetyl-D-glucosamine supplemented selective medium. $\times 125\ 000$ (original magnification).

Tube titration of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of amoxycillin for *H influenzae*

	Tube										Control
	1	2	3	4	5	6	7	8	9	10	
Amoxycillin concentration (mg/l)	50.0	25.0	12.5	6.25	3.125	1.56	0.8	0.4	0.2	0.1	Nil
Turbidity*	0	0	0	0	0	0	⊠	+++	+++	+++	+++
Subculture on <i>N</i> -acetyl-D-glucosamine medium*	0	0	0	0	⊠	+	+++	+++	+++	+++	+++
Morphology by light microscopy†	N	N	N	N	S±	S+	S++	S++	S±	N	N

* 0 = None, + = light growth, +++ = heavy growth. ⊠ = MIC. ⊞ = MBC. † N = Normal. S = Spheroplastic (± = scanty, + = moderate numbers, ++ = many).

inhibitory concentration of amoxycillin for the organism was 0.8 mg/l. Subculture of the tube contents on to solid media containing *N*-acetyl-D-glucosamine resulted in growth of organisms from tubes containing dilutions of antibiotic up to, but not including, 3.12 mg/l (minimal bactericidal concentration).

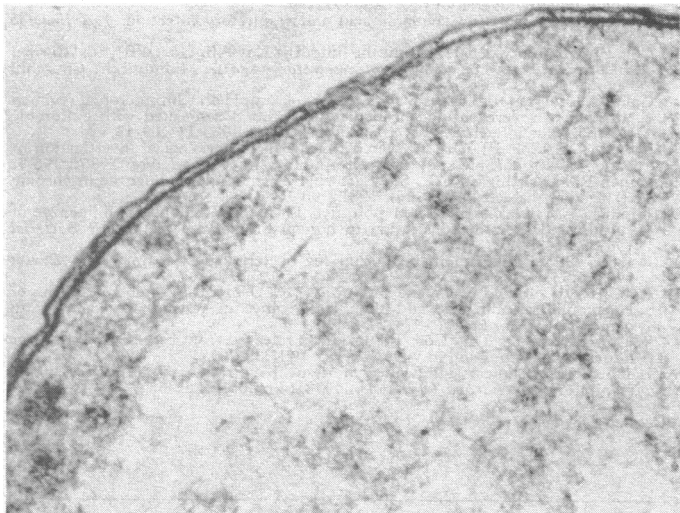


FIG 3—Transmission electronmicrograph of spheroplast of *H influenzae* induced in vitro by minimal inhibitory concentration of amoxycillin for organism. $\times 125\ 000$ (original magnification).

Transmission electron microscopy—Examination of the spheroplasts by transmission electron microscopy (fig 2) showed very large organisms with visible cell walls similar to the appearance of spheroplasts induced by exposure to amoxycillin in vitro (fig 3).

Discussion

Most of the patients in this study were suffering from bronchiectasis, and some from cystic fibrosis. They had received prolonged courses of various antibiotics, often given parenterally or by inhalation, or both, which tended to prevent bacterial isolation. Despite this, several spheroplastic forms were isolated by the use of medium supplemented with *N*-acetyl-D-glucosamine. Such organisms would doubtless not have been recognised otherwise. It is also likely that some *H influenzae* isolated on conventional medium had reverted from the spheroplastic phase on primary culture.

So far as we know this primary isolation of colony forming spheroplastic *H influenzae* from patients is a new finding, although they have been observed microscopically in sputum and induced experimentally in vitro.¹¹ We suspect that the number of such cultures might be greater from untreated patients and from those treated with conventional doses of oral antibiotic. On the other hand, the antibiotic treatment of the patients may have encouraged the emergence of mutants with defective cell walls. To try to resolve this problem a study of the prevalence of carriage of *H influenzae* and its spheroplastic mutant form in an unselected population in the community is in progress. If this confirms the findings there will be a compelling case for the routine addition of *N*-acetyl-D-glucosamine to selective medium for the culture of *H influenzae*. Certainly nine isolates would have been missed without using such medium, and in that group the highest proportion of spheroplastic organisms was found—an incidence which might have been higher but for probable rapid reversion of such mutant forms to the intact state.

Lysozyme dissolves the mucopeptide responsible for rigidity of the bacterial cell wall by cleaving the acetylglucosamine from the acetyl muramic acid molecules. Penicillin inhibits the metabolism of a complex nucleotide (uridine diphosphate-*N*-

acetyl muramic acid pentapeptide) into peptidoglycan. This entails transfer of the *N*-acetyl muramic acid pentapeptide from the nucleotide linkage to a lipid component of the cytoplasmic membrane followed by the addition of *N*-acetyl-D-glucosamine on transference of the whole unit into the growing bacterial cell wall—by translocation from middle to outer surface of the cell membrane. Both lysozyme and penicillin thereby exert an effect on bacterial cell wall synthesis, and it is possible that both have an equal or even synergistic role in the formation of spheroplastic bacteria in the human respiratory tract when the patient receives β lactam antimicrobial chemotherapy.

It is of considerable clinical importance in relation to chronic bronchial infection that spheroplasts are likely to be induced by β lactam antibiotics—in our in vitro titration by concentrations of amoxycillin ranging from 0.4 mg to 1.56 mg/l. Conventional doses of this antibiotic—for example, 250 mg by mouth four times a day—achieve at best sputum concentrations of approximately 1 mg/l and are therefore unlikely to destroy spheroplasts of *H influenzae* and may actually induce them in vivo. The transmission electronmicroscopical studies are important in this regard because they showed these abnormal forms to possess a cell wall. One interpretation is that the antibiotic, in the unsatisfactory concentration present in the respiratory tract achieved by conventional oral dosage, only weakened the cell wall, allowing distortion to an abnormally large form—but not to the point where it became sufficiently deficient to lyse through osmotic processes.

Most Gram negative rods—for example, *Escherichia coli*—burst from raised internal pressure when their cell walls are weakened by β lactam antibiotics. *H influenzae* differs in possessing the ability to form spheroplasts which are osmotically stable. This was reported by Roberts *et al*,¹³ who made serial dilutions of amoxycillin in two different broths of known osmolality. The higher osmolality corresponded with that of bronchial secretions, whereas that of the second was lower. After inoculation with *H influenzae* the broths were incubated and the tubes showed turbidity. When examined by light microscopy both media were seen to contain spheroplasts, lysis not having occurred. *H influenzae* spheroplasts are therefore capable of survival in media of low osmolality (certainly in bronchial secretions) and do not require an osmotic stabiliser to prevent lysis. Lysis of these spheroplastic forms may require considerably higher concentrations of antibiotics and would explain why in our in vitro sensitivity titration such forms were not seen in concentrations of amoxycillin above 3 mg/l.

In chronic bronchial infection such as bronchiectasis, as opposed to “classical” acute infection, the affected part of the bronchial tree becomes damaged by an initial insult leading to obstruction and infection. Thereafter colonising microorganisms compose a (usually) non-invasive, avirulent flora to which the patient mounts exuberant local and systemic immunological and non-specific responses limiting the disease to the lung (metastatic infection is extremely rare). This host response against the microbial flora is damaging to tissue and, together with damaging substances released locally by certain bacteria,¹⁴ causes a vicious circle of progressive bronchial damage resulting in worsening symptoms and, in some patients, rapid deterioration and death.¹ The principles of effective treatment are therefore to achieve truly bactericidal concentrations of antibiotic at the site of the lesion to reduce or eliminate the “microbial load” and (possibly) suppress the unwanted tissue damaging part of the host response.¹ But, unlike in acute infection in previously normal lung where blood supply is normal or increased, probably in chronic bronchial infection—for example, bronchiectasis—vascular access of drugs to the shrunken, scarred site of the lesion is poor.¹⁵ This together with thick, purulent secretions within the airways demands a higher serum concentration of antibiotic to allow penetration to the site of the progressive destructive lesion—and this correspondingly requires appropriate—that is, higher—oral dosage of antibiotic.

Based on that rationale appropriate dose antimicrobial chemotherapy in severe bronchiectasis has shown encouraging

remission of symptoms and signs in about 60% of patients both acutely^{1,8} and on follow up.^{1,3} More recently, the first indications that this form of treatment may slow or halt progression of disease have been seen.¹ It is therefore of considerable interest to find that the spheroplasts of *H influenzae* studied here possessed a cell wall and that this was not completely defective (L forms) as had been assumed.^{5,11,13} Appropriate dose antibiotic treatment may well inhibit formation or even destroy the reservoir of such "persister" spheroplasts, and this may be one reason for the success of this approach. In the context it is notable that rapid (within a few days) return of pus in sputum after stopping conventional antibiotic treatment is associated with much more frequent isolation (70%) of *H influenzae*, whereas late recurrence (weeks or months) is associated equally with *H influenzae* (55%) and *Streptococcus pneumoniae* (55%).²

It is possible that spheroplastic *H influenzae* contain tissue damaging substances and, if this is the case, it may be important to inhibit their formation by using a dose of antibiotic that will achieve a bactericidal concentration in the secretions—not merely a bacteriostatic concentration, which may encourage emergence of such forms.

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Voice changes after thyroidectomy: role of the external laryngeal nerve

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Abstract

Hitherto voice changes have been regarded as an infrequent complication of thyroidectomy and damage to the recurrent laryngeal nerve has been given as their major cause. Voice function was assessed in 325 patients after thyroidectomy. Permanent changes occurred in 35 (25%) after subtotal thyroidectomy and in 19 (11%) after lobectomy. The commonest cause of voice change appeared to be injury to the external laryngeal nerves on one or both sides. Damage to the recurrent laryngeal nerve, which was routinely identified and protected, was rarely a cause. When the external laryngeal nerves were identified and preserved, permanent voice changes occurred in only 5% of cases; this was similar to the incidence of 3% in controls after endotracheal intubation alone.

The course of the external laryngeal nerve is variable,

and consequently mass ligation of the vessels at the top of the upper pole will damage it in a high proportion of cases. To minimise this serious complication these nerves should be identified and protected as well as the recurrent nerves and voice function should be assessed early in the postoperative period by laryngoscopy and by a speech therapist.

Introduction

The safety of thyroidectomy has been enhanced by anaesthetic refinements and preoperative control of the thyroid disease, so permitting unhurried, precise surgery in euthyroid patients. Nevertheless, a few patients are affected postoperatively by hypoparathyroidism, hypothyroidism, or change in the voice. Voice changes range from slight huskiness, poor volume, and a tired voice by evening to those necessitating a complete change of leisure and professional activities.

Voice change after thyroidectomy has generally been held to be the result of damage to the recurrent laryngeal nerve. Thus identification and preservation of this nerve is widely practised.¹ Some surgeons deliberately avoid dissecting the recurrent nerve, but Roy *et al* found unilateral damage to the recurrent laryngeal nerve in 13.3% of 300 such cases.² Painter, reviewing operations for toxic goitre, concluded that "the integrity of the recurrent laryngeal nerves is not the only factor in the preservation of a normal voice after operation,"³ and Nash maintained that after thyroidectomy "a considerable number suffer a reduction of voice range."⁴ We encountered a disturbingly high incidence of postoperative voice changes and therefore reviewed a series of 415 patients after thyroidectomy.

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