Growth-Enhancing Effects of Culture Filtrates of Sputum Isolates on the L-Forms of *Haemophilus influenzae*

HARUMI SHISHIDO

Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852

SHISHIDO, H. Growth-Enhancing Effects of Culture Filtrates of Sputum Isolates on the L-Forms of *Haemophilus influenzae*. Tohoku J. exp. Med., 1986, 149 (3), 271-282 — The growth-enhancing effects of culture filtrates of respiratory pathogenic bacteria, including *Haemophilus influenzae*, as well as normal floral bacteria other than *Neisseria perflava* and *Branhamella catarrhalis* on L-forms of *H. influenzae* were examined in vitro, using five species of major respiratory pathogenic bacteria and seven species of normal floral bacteria commonly isolated from the sputum of patients with chronic respiratory tract infections. The growth-enhancing factor(s) was present in the filtrates prepared from the culture of respiratory pathogenic *Streptococcus pneumoniae* (2) and *Staphylococcus aureus* (1), the effects of which were as potent as those of a culture filtrate of *B. catarrhalis* used as the positive control. The culture filtrates of respiratory pathogenic *Pseudomonas aeruginosa* (2) and *Klebsiella pneumoniae* (1) had weak growth-enhancing effects on *H. influenzae* L-forms. The culture filtrates of 21 strains of normal floral bacteria isolated from the sputum including α-hemolytic Streptococci (4), non-hemolytic Streptococci (4), *Micrococcus roseus* (5), coagulase-negative Staphylococci (3), and *Neisseria* spp. (5) had growth-enhancing effects on *H. influenzae* L-forms. These data elucidate the significance of *H. influenzae* L-forms in patients with chronic respiratory tract infections. —— *Haemophilus influenzae* L-forms; respiratory pathogenic bacteria; normal floral bacteria isolated from the sputum; recurrent infections due to *H. influenzae*; L-forms growth-enhancing factors

In an accompanying paper (Shishido et al. 1986), we discussed the presence of *Haemophilus influenzae* L-forms growth-enhancing factors in culture filtrates of two bacterial species, *Neisseria perflava* and *Branhamella catarrhalis*, isolated from sputum. We suggested the possibility that these culture filtrates may be important clues to understanding the correlation between the recurrent infections due to *H. influenzae* and the entire process of induction, growth and reversion of *H.*
influenzae L-forms. Isolates from sputum of patients with chronic respiratory tract infections with recurrent H. influenzae infections may provide a favorable environment for L-forms of H. influenzae, in vivo.

For clarification, the growth-enhancing effects of various culture filtrates of respiratory pathogenic bacteria, including H. influenzae, as well as normal floral bacteria in the upper respiratory tract (other than N. perflava and B. catarrhalis) were examined in vitro, using five species of major respiratory pathogenic bacteria and seven species of normal floral bacteria commonly isolated from the sputum of patients with chronic respiratory tract infections.

In this paper, the term “L-forms” refers to a continuously growing bacterial organism with evidence of cell wall defect (Shishido et al. 1986).

**Materials and Methods**

L-forms of H. influenzae. The strain NNHL841 of H. influenzae L-form used in the in vitro experiments in the present study was isolated from the cerebrospinal fluid of a 14-year-old boy with meningitis, in the laboratory of the Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University. Hypertonic Supplemented Peptone Broth (HSPB; Becton-Dickinson, Rutherford, NJ, USA) supplemented with N. perflava NN34 culture filtrates was used for the primary isolation. The L-form of H. influenzae NNHL841 was isolated as the primary culture, followed by three consecutive subcultures to HSPB supplemented with a culture filtrate of N. perflava NN34 or that of B. catarrhalis NNBR44, and was kept at −70°C until use. The inoculum of H. influenzae L-forms NNHL841 was viable enough to accomplish all the experiments described herein, for at least 18 months. The revertants from H. influenzae L-forms NNHL841 were serotyped as type b, were biotyped into type 1, and did not produce β-lactamases.

Filtrability of H. influenzae L-forms. Filtrability of L-forms of H. influenzae NNHL841 was determined by using membrane filters with pore sizes of 0.05 μm, 0.10 μm, 0.22 μm, and 0.45 μm (Millipore Corporation, Bedford, MA, USA). After passage through a membrane filter of each pore size, a filtrate was inoculated into HSPB supplemented with B. catarrhalis NNBR44 culture filtrates and then subcultured on a brain heart infusion agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with hemin (Wako Pure Chemical Industries, Ltd., Osaka) and β-nicotinamide adenine dinucleotide (β-NAD; Sigma Chemical Co., St. Louis, MO, USA) at final concentrations, respectively, of 20 μg and 4 μg per ml. A viable revertant colony was considered as a filtrable growing bacterium.

Transmission electron microscopy. The organisms of H. influenzae L-forms NNHL841 were harvested after 22 hr of incubation at 37°C in a medium consisting of HSPB supplemented with B. catarrhalis NNBR44 culture filtrates by centrifugation at 3,000 × g at 4°C for 20 min and prefixed with 2% glutaraldehyde in 0.01 M sodium cacodylate, pH 7.0, for 18 hr. Postfixation was performed in 0.75% osmium tetroxide in cacodylate buffer for 18 hr at 4°C. After dehydration in a graded series of ethanol solutions, the fixed specimens were embedded in Epon 812. Sections were cut with a Reichert Ultra Cut E and stained with 8% uranyl acetate and lead salts. The specimens were examined with a JEM 100CX operated at 80 kV.

Preparation of culture filtrates. The filtrates of a culture were prepared, as described in an accompanying paper (Shishido et al. 1986), from each of 31 strains tested, using brain heart infusion broth (BBL Microbiology Systems, Cockeysville, MD, USA) to which 5% of defibrinated horse blood was added, the preparation centrifuged at 3,000 × g then passed through a membrane filter with a pore size of 45 μm or 0.2 μm (Gelman Science, Inc., MI, USA). The membrane filter of 0.2 μm pores was used only for α-hemolytic Streptococci,
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which could pass through 0.45 μm pores. These strains, which had been isolated in the Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, consisted of five species of respiratory pathogenic bacteria and seven species of normal floral bacteria isolated from the sputum of the patients with chronic respiratory tract infections (CRTIs). The respiratory pathogenic bacteria included four strains of *H. influenzae*, two strains of *S. pneumoniae*, one strain of *S. aureus*, two strains of *P. aeruginosa*, and one strain of *K. pneumoniae*. The normal floral bacteria included four strains of α-hemolytic Streptococci (4 *Streptococcus mitis*), four strains of non-hemolytic Streptococci (4 *Streptococcus salivarius*), five strains of *Micrococcus roseus*, five strains of coagulase-negative Staphylococci (2 *Staphylococcus epidermidis*, 1 *Staphylococcus haemolyticus*), and five strains of *Neisseria* spp. (4 *Neisseria elongata*, 1 *Neisseria subflava*).

Effects of culture filtrates on growth and reversion of *H. influenzae* L-forms. The presence or absence of the growth-enhancing factor(s) in each culture filtrate was determined by a growth curve of the viable count of revertants from L-forms of *H. influenzae*, as described elsewhere (Shishido et al. 1986), using brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md, USA) supplemented with hemin and β-NAD at final concentrations, respectively, of 20 μg/ml and 4 μg/ml as a counting medium for the revertants. The inoculates of L-forms in all the experiments in the present study were *H. influenzae* L-forms NNHL 841 which had been kept at −70°C until each experiment. A medium contained HSPB and a culture filtrate prepared from each of 31 strains examined to a concentration of 5%. A culture filtrate of *B. catarrhalis* NNBR44 was used as a positive control and plain culture media was the negative control. A revertant colony considered as "a large colony" was counted in a viable revertant (Shishido et al. 1986). All the experiments were performed using a medium supplemented with the culture filtrates of each bacterial strain examined, a positive control, and a negative control, in parallel cultures.

**Results**

**Filtrability of *H. influenzae* L-forms.** The filtrates of *H. influenzae* L-forms NNHL841 passed through a membrane filter with a pore size of 0.22 μm or less yielded no revertants, but those through a filter with a pore size of 0.45 μm were capable of growth of viable revertants, as these filtrates were subcultured to HSPB supplemented with *B. catarrhalis* culture filtrates and then subcultured on brain heart infusion agar supplemented with hemin and β-NAD. A control experiment demonstrated that the parent forms of respiratory pathogenic *H. influenzae* could not pass through a 0.45 μm membrane filter, as a viable bacterium.

**Transmission electron microscopy.** Morphology of transmission electron micrographs of the organisms of *H. influenzae* L-forms NNHL841 is shown in Fig. 1. The L-form of *H. influenzae* was apparently intact with visible cell walls but the size of the L-form bacteria was much larger than that of the parent bacterial forms and the shape of the L-forms was spherical. Therefore, the cell wall of the L-forms of *H. influenzae* must be so defective that this bacterial form could maintain a very large spherical form in size and shape.

**Effects of culture filtrates of respiratory pathogenic bacteria on *H. influenzae* L-forms.** None of the culture filtrates prepared from four strains of respiratory pathogenic *H. influenzae* demonstrated growth-enhancing effects on the L-forms of *H. influenzae* (Fig. 2). The growth-enhancing factor(s) was present in the
filtrates prepared from two strains of respiratory pathogenic *S. pneumoniae* (Fig. 3) and from one strain of respiratory pathogenic *S. aureus* (Fig. 4), the effects of which were as potent as those of a culture filtrate of *B. catarrhalis* NNBR44 used as the positive control. The culture filtrates of two strains of respiratory pathogenic *P. aeruginosa* (Fig. 5) and one strain of respiratory pathogenic *K. pneumoniae* (Fig. 6) had weak growth-enhancing effects on the growth and reversion of *H. influenzae* L-forms. All of these growth curves of the viable revertants

![Transmission electronmicrograph of L-forms of *H. influenzae* NNHL841 examined after 22 hr of incubation at 37°C in a medium consisting of HSPB supplemented with *B. catarrhalis* NNBR44 culture filtrates. Apparently intact, visible cell walls were observed in large spherical L-form bacterial cells. (A) Original magnification, ×48,000. (B) Original magnification, ×100,000.](image)
Fig. 2. Enhancing effects of culture filtrates of four strains of *H. influenzae* on growth and reversion of the L-forms of *H. influenzae*. •, adding filtrates of a culture of *H. influenzae* NNH1; ■, *H. influenzae* NNH2; ▲, *H. influenzae* NNH3; ▼, *H. influenzae* NNH4; ○, positive control; △, negative control.

Fig. 3. Enhancing effects of culture filtrates of two strains of *S. pneumoniae* on growth and reversion of the L-forms of *H. influenzae*. •, adding filtrates of a culture of *S. pneumoniae* NNP1; ■, *S. pneumoniae* NNP2; ○, positive control; △, negative control.
from L-forms of *H. influenzae* were situated at the middle of a negative control and a positive control, in each experiment.

**Effects of culture filtrates of normal floral bacteria from the sputum on *H. influenzae* L-forms.** The effects of culture filtrates prepared from four strains of α-hemolytic Streptococci, four strains of non-hemolytic Streptococci, five strains of Staphylococcus, and five strains of Pseudomonas were studied. The results are summarized in Table 1.

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**Fig. 4.** Enhancing effects of culture filtrates of a strain of *S. aureus* on growth and reversion of the L-forms of *H. influenzae*. ●, adding filtrates of a culture of *S. aureus* NN1; ○, positive control; △, negative control.

**Fig. 5.** Enhancing effects of culture filtrates of two strains of *P. aeruginosa* on growth and reversion of the L-forms of *H. influenzae*. ●, adding filtrates of a culture of *P. aeruginosa* NNα1; ■, *P. aeruginosa* NNα2; ○, positive control; △, negative control.
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of \textit{Micrococcus roseus}, three strains of coagulase-negative Staphylococci, and five strains of Neisseria spp. on the growth and reversion of \textit{H. influenzae} L-forms are shown in Figs. 7, 8, 9, 10 and 11, respectively. The culture filtrates of all of these 21 strains isolated from the sputum of the patients with CRTIs had growth-
Fig. 8. Enhancing effects of culture filtrates of four strains of non-hemolytic Streptococci on growth and reversion of L-forms of *H. influenzae*. •, adding filtrates of a culture of *S. salivarius* NN2; ■, *S. salivarius* NN5; ▲, *S. salivarius* NN1; ○, *S. salivarius* NN3; ◊, positive control; △, negative control.

Fig. 9. Enhancing effects of culture filtrates of five strains of *M. roseus* on growth and reversion of L-forms of *H. influenzae*. •, adding filtrates of a culture of *M. roseus* NN5; ■, *M. roseus* NN1; ▲, *M. roseus* NN4; ○, *M. roseus* NN3; ×, *M. roseus* NN2; ◊, positive control; △, negative control.
Fig. 10. Enhancing effects of culture filtrates of three strains of coagulase-negative Staphylococci on growth and reversion of L-forms of *H. influenzae*. •, adding filtrates of a culture of *S. epidermidis* NN1; ■, *S. epidermidis* NN5; ▲, *S. haemolyticus* NN2; ○, positive control; △, negative control.

Fig. 11. Enhancing effects of culture filtrates of five strains of *Neisseria* spp. on growth and reversion of L-forms of *H. influenzae*. •, adding filtrates of a culture of *N. elongata* NNNE3; ■, *N. elongata* NNNE1; ▲, *N. subflava* NNNE4; ▼, *N. elongata* NNNE5; ×, *N. elongata* NNNE2; ○, positive control; △, negative control.
enhancing effects on the L-forms of *H. influenzae*.

**DISCUSSION**

The L-forms of *H. influenzae* in the present study are thought to be essentially the same bacterial forms described by Roberts et al. (Roberts 1984; Roberts et al. 1984) as "spheroplastic forms" and by Yourassowsky et al. (1973) as "unstable L-forms". Both reports indicated that these bacterial forms possessed cell walls demonstrated by the transmission electronmicroscopical examination. As shown in Fig. 1, the strain NNHL841 of *H. influenzae* L-form used in the present study had a visible cell wall and a very large spherical form. On the other hand, Madoff (1976) described that ultrathin sections demonstrated the structure of L-forms of *H. influenzae* which had been induced in vitro in his laboratory to be completely devoid of cell wall. The L-forms in the present study were so flexible that they could pass through a membrane filter with 0.45 μm pores through which the parent bacteria could not. However, they were unable to pass through a 0.22 μm pore, although the L-forms, or spheroplasts, of other species of bacteria were reported to pass through the membrane filters with pore sizes smaller than 0.22 μm (Okazaki et al. 1981). These findings of the filtrability of viable L-forms were considered to be consistent with the transmission electron microscopical findings that the L-forms of *H. influenzae* possess visible cell walls.

The data obtained in the present study elucidate the significance of L-forms of *H. influenzae* in recurrent infections due to *H. influenzae* in patients with CRTIs. *H. influenzae* is most frequently associated with recurrent infections in CRTIs and is usually isolated from the sputum of the patients with CRTIs, accompanied by isolations of a number of normal floral bacteria and/or other respiratory pathogenic bacteria (Uzuka et al. 1983; Cole 1984). Want and May (1975) described preliminary observations on the behaviour of L-forms of *H. influenzae* in the environment provided by unsterilized mucoid sputum obtained from patients with chronic bronchitis. L-forms of *H. influenzae* were reported to have been induced on a medium in which mucoid sputum formed the sole source of nutrients, and remained viable for at least 48 hr when an osmotic stabiliser was not added. A mucoid sputum may contain the L-form growth-enhancing factors demonstrated in these culture filtrates of *S. pneumoniae*, *S. aureus*, *B. catarrhalis*, and normal floral bacteria in the upper respiratory tracts, which were isolated from the sputum, in the present study.

Roberts et al. (1984) reported that N-acetyl-d-glucosamine promoted the growth of spheroplasts of *H. influenzae*, whereas N-acetyl muramic acid did not and that the selective medium supplemented with N-acetyl-d-glucosamine enhanced primary isolation of colony forming, spheroplastic *H. influenzae*, which reverted to normal bacteria on subculture. The effects of the growth-enhancing factors demonstrated in the present study would be similar to those of N-acetyl-d-glucosamine. It is postulated that the growth-enhancing factors excreted into the
medium by the various bacteria mentioned above may contain N-acetyl-D-glucosamine, probably with some cross-linking peptides. This idea is based on the biochemical analysis of *B. catarrhalis* culture filtrates which revealed that the growth-enhancing factors to L-forms of *H. influenzae* were several peptides with a molecular weight of 1,000 to 5,000 (Shishido et al. 1986), and on the basis of the cell wall components containing glycan strands composed of two alternating amino sugars, peptide subunits, and peptide cross-linking bridges (Wilson and Miles 1975). The results in the present study suggest that these growth-enhancing factors are more likely to be produced by gram-positive cocci, Streptococci, Staphylococci, and Micrococci, than by gram-negative bacilli, *H. influenzae*, *K. pneumoniae* and *P. aeruginosa*. Thus, the growth-enhancing factor(s) probably depends on cell wall components of each bacterium, among which certain peptides might play an important role in promoting or enhancing effects on the growth and reversion of *H. influenzae* L-forms.

De Castro-Costa and Landman (1977) proposed that L-forms of *Bacillus subtilis* produced a reversion inhibitory factor which was nondialyzable and sensitive to trypsin, heat and detergent. This inhibitor had the properties of a protein and was suggested to be the autolysin of the parent bacterium which inhibited the re-establishment of peptidoglycan. Dienes (1970) reported that L-forms of *H. influenzae* reverted to parent bacteria under the influence of either a culture or the filtrate of a culture of Bacillus sp. These reported effects of Bacillus sp. on L-forms reversion provided another explanation for the data obtained in the present study. Further studies are underway to clarify the essential substance of the growth-enhancing factor(s) and their site(s) of action in vitro.

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References


