Survival of nontypeable \textit{Haemophilus influenzae} in macrophages

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Abstract

In this study we have investigated the ability of nontypeable, nonencapsulated \textit{Haemophilus influenzae}, NT477 to survive in the J774 mouse macrophage-like cell line. Viable, intracellular nontypeable \textit{H. influenzae} could still be recovered from macrophages 72 h after phagocytosis. In contrast, \textit{H. influenzae} strain Rd, an avirulent, nonencapsulated variant of a serotype d strain, was killed within 24 h. These differences suggest that NT477, in comparison to Rd, possesses unique attributes that enable it to survive in macrophages for prolonged periods. To determine whether this trait is ubiquitous amongst nontypeable \textit{H. influenzae}, 33 primary clinical isolates obtained from children with otitis media were screened for their ability to survive in macrophages. Of these isolates, 82\% were able to persist in an intracellular environment for periods of at least 24 h. The number of viable organisms recovered at this time ranged from 2 \times 10^4 to 50 colony-forming units per strain indicating that the extent to which nontypeable \textit{H. influenzae} can resist macrophage-mediated killing varies between strains. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Intracellular survival; Macrophage; \textit{Haemophilus influenzae}

1. Introduction

Nonencapsulated, nontypeable \textit{Haemophilus influenzae} (NTHi) strains are a major cause of both upper and lower respiratory tract infections in humans. Pre-school children and individuals with an underlying disease which affects the innate mucosal immune system, such as cystic fibrosis, are particularly vulnerable [1,2]. NTHi are also responsible for over a quarter of the cases of acute otitis media, an inflammation of the middle ear. The peak age of incidence for this disease is between 6 and 24 months, and by 3 years of age approximately 70\% of children will have had at least one attack [1,3]. One of the features of such infections is that they often recur once antibiotic treatment has ceased. This results in middle ear effusions which last for weeks, months or even years, and can cause severe hearing impairment [4]. In the majority of children, otitis media infections resolve without antibiotic treatment in about 3 days. However in those children which require, but fail to respond to, antimicrobial therapy it has been shown that early recurrent NTHi otitis media is usually caused by the initial infecting strain [5]. Indeed, in otitis-prone children, some strains of NTHi have been shown to re-colonise the nasopharynx after an absence of up to 11 months, despite intervening antibiotic treatment [6]. Such observations have lead to the suggestion that NTHi may have an intracellular reservoir within the host where it is protected from host immune defences and circulating antibiotics. Recurrent infection is not restricted to otitis media, and persistence of NTHi in the lower respiratory tract of patients with either acute or chronic respiratory infections is also an important problem [7].

Although not traditionally thought of as an intracellular pathogen, NTHi appear capable of entering and surviving within Chang epithelial cells [8]. Intracellular NTHi have also been observed in artificially infected adenoidal tissue in organ culture [9] using scanning electron microscopy. Bacteria were observed in clusters between adjacent cells and in the phagocytic vacuoles of mononuclear cells. In additional studies, adenoids removed from children with adenoid hypertrophy were also found to harbour NTHi. Bacteria were found in the reticular crypt epithelium and in macrophage-like cells located in the subepithelium. Notably, the organisms in macrophage-like cells were viable, suggesting that NTHi may target such cells to obtain an intracellular niche [10].

To investigate the hypothesis that NTHi can survive in...
and incubated overnight at 37°C with 5% CO2. Prior to
incubation, the monolayers were washed three times in
PBS to remove extracellular bacteria and fresh DMEM
containing 10% FBS and polymyxin B sulfate (10 μg
ml−1) added. This concentration of antibiotic had previ-
ously been shown to kill 100% of a 5 × 10^8 suspension of
H. influenzae in 1 h. Incubation was continued at 37°C,
5% CO2, and at various time points monolayers were
washed three times in PBS, lysed with sterile water, and
the number of internal bacteria enumerated by plating serial
dilutions of the lysate onto BHI plates.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All nontypeable H. influenzae strains were primary otitis
media isolates obtained from Dr J. Eskola (National Pub-
lic Health Institute, Helsinki, Finland). H. influenzae strain
Rd is an avirulent, spontaneous capsule-deficient mutant
of a serotype d strain. H. influenzae strains were grown in
brain–heart infusion broth (BHI) supplemented with hae-
min (10 μg ml−1) and NAD (2 μg ml−1). Solid BHI me-
dium was prepared by the addition of Bacto agar 1.5%
(w/v) and 10% (v/v) Levinthals base [11]. To test for sen-
sitivity to polymyxin B sulfate liquid cultures of NTHi
strain Rd, an avirulent, spontaneous, nonencapsulated
strain of a serotype d strain, was killed by macro-
phages within 24 h of phagocytosis. Evidence is presented
that this attribute is not restricted to NT477 and that the
ability to survive in macrophages is prevalent amongst
NTHi.

2.2. Cell lines

A phagocytic BALB/c mouse macrophage-like cell line
J774A.1 (ATCC: 91051511) was used. Cells were grown in
Dulbecco’s modified Eagle medium (DMEM) supple-
mented with 10% (v/v) heat-inactivated foetal bovine se-
rum (FBS) (Gibco, BRL), 1 mM L-glutamine, 1 mM es-
tenential amino acids and 1 mM sodium pyruvate (Sigma).
Monolayers were maintained at 37°C in a 5% CO2 moist
chamber and passaged every 3–5 days.

2.3. Invasion and persistence of bacteria in
cultured cell lines

For invasion assays, cells were seeded into 6-well tissue
culture plates (Costar) at approximately 2 × 10^6 cells/well
and incubated overnight at 37°C with 5% CO2. Prior to
bacterial infection the monolayers were washed twice in
sterile phosphate-buffered saline (PBS) before the addition
of fresh DMEM without FBS. Mid-exponential-phase bac-
teria from freshly growing cultures were harvested and
resuspended in DMEM, and 100 μl was inoculated to each
well to give a multiplicity of infection of approxi-
ately 100:1. Infected monolayers were then incubated
for 1 h at 37°C in 5% CO2 to allow phagocytosis to occur.
After this period, monolayers were washed three times in
PBS to remove extracellular bacteria and fresh DMEM
containing 10% FBS and polymyxin B sulfate (10 μg
ml−1) added. This concentration of antibiotic had previ-
ously been shown to kill 100% of a 5 × 10^8 suspension of
H. influenzae in 1 h. Incubation was continued at 37°C,
5% CO2, and at various time points monolayers were
washed three times in PBS, lysed with sterile water, and
the number of internal bacteria enumerated by plating serial
dilutions of the lysate onto BHI plates.

2.4. Immunofluorescence staining and microscopy of
infected monolayers

The monoclonal antibody MAHi 419, kindly provided
by S. Borrelli (Karolinska Institute, Sweden), was used to
detect NTHi. This Mab binds to NTHi and Rd lipopoly-
saccharides (LPS) in a nonphase variable manner, as
judged by colony immunoblotting (data not shown).

For immunofluorescence staining of infected mono-
layers, infection was carried out as described above, the
only difference being that macrophage monolayers were
cultivated on glass coverslips, inside tissue-culture wells.

After the desired incubation period, monolayers were
washed three times with PBS and the coverslips were care-
fully removed from the wells. The macrophages were then
permeabilised by submerging the coverslip into acetone
(pre-cooled to −20°C) for 3 min. After air drying, the
monolayers were blocked in PBS containing 3% (w/v) bo-
vine serum albumin (PBS–BSA), followed by incubation
with Mab MAHi 419 for 1 h at room temperature in a
humid chamber. Coverslips were then carefully washed in
PBS before incubation with a fluorescein isothiocyanate
(FITC)-conjugated rabbit anti-mouse antibody (Sigma),
diluted 1 in 20 in PBS–BSA, for 30 min. Coverslips were
mounted on microscope slides using Vectashield® mount-
ing medium (Vector Laboratories Inc.) and sealed with a
clear varnish. The preparations were viewed at a magnifi-
cation of ×100 in a LEICA DMR epifluorescence micro-
scope.

3. Results

3.1. Long-term survival of NT477 in J774A.1

To assess the ability of NTHi to invade and survive in
macrophages, the J774 macrophage cell line was infected
with strain NT477, and the ability of this organism to
survive intracellularly compared with that of H. influenzae
strain Rd, an avirulent, spontaneous, nonencapsulated
mutant of a serotype d strain. The latter strain provided
a measure of the killing capacity of the J774 macrophages,
indicating that the macrophages used in this study pos-
sessed bactericidal activity and were able to kill a non-
pathogenic strain of *H. influenzae*. As illustrated in Fig. 1, at 0 h the number of cell-associated bacteria, including both intra- and extracellular bacteria, was very similar for both organisms, representing between 5 and 10% of the original inoculum. To distinguish between intra- and extracellular populations of bacteria, the incubation was continued in the presence of polymyxin B sulfate, which selectively kills extracellular bacteria. When used at a concentration of 10 μg ml⁻¹, polymyxin B sulfate kills 100% of a 5×10⁸ suspension of *H. influenzae* in 1 h (data not shown). 1 h after the addition of polymyxin B sulfate, a distinct difference in the number of viable intracellular bacteria was observed between NT477- and Rd-infected cells. This difference continued to increase in magnitude until, by 24 h, no viable intracellular Rd remained. Although 90% of NTHi were killed by macrophages by 24 h, we were still able to recover longer viable at this time point. Distinct differences in the number of viable intracellular bacteria was observed between NT477- and Rd-infected cells. This difference continued to increase in magnitude until, by 24 h, no viable intracellular Rd remained. Although 90% of NTHi were killed by macrophages by 24 h, we were still able to recover ~10⁵ colony-forming units (cfus) per well at this time point, and these organisms continued to persist in the macrophage for a further 48 h. The relative persistence of NT477 and Rd in macrophages was also monitored by fluorescence microscopy over a 24-h period. To visualise intracellular bacteria, infected macrophages were permeabilised and probed sequentially with MAHi 416, a monoclonal antibody specific for *H. influenzae* LPS, followed by a FITC-conjugated rabbit anti-mouse antibody (Fig. 2). In the absence of a permeabilisation step, bacteria were not FITC labelled, confirming that the organisms visualised by this technique are intracellular, with the exception of the 0-h time point where many macrophage-associated bacteria were extracellular. 2 h after the addition of polymyxin B sulfate, many intracellular bacteria can be observed grouped in clusters around the nucleus. Over the next 6 h, the number of intracellular organisms gradually decreased, suggesting that phagocytosed organisms were being digested and gradually expelled by the cell. Consistent with the intracellular survival assay, NT477 were still present in macrophages at 24 h. In contrast, at the same time point no intracellular Rd were detected (data not shown), which indicates that the macrophages cell line used in this study is capable of killing an avirulent strain of *H. influenzae*.

### 3.2. Is the ability to survive in macrophages a ubiquitous trait in NTHi clinical isolates?

To determine whether the ability to survive in macrophages is ubiquitous amongst NTHi isolates and not unique to NT477, we examined the uptake and intracellular survival properties of 33 NTHi clinical isolates in J774A.1. The results are shown in Fig. 3. On comparison of the 0-h time points, distinct differences in the numbers of cell-associated organisms can be seen amongst the clinical isolates tested. For example, the number of cell-associated bacteria recorded for strains NT886, 1542, 1003 and 1233 is 10-fold less than many of the other strains tested. These differences may reflect variation in the ability of NTHi isolates to either adhere to, or be phagocytosed by, macrophages. At 24 h post-infection, 84% of all isolates tested were recovered from macrophages, indicating that the majority of NTHi are able to persist in J774A.1. Only strains NT487, 916, 279, 608, 1151 and 1124 were no longer viable at this time point. Distinct differences in the number of organisms recovered after 24 h were observed amongst isolates that could survive in macrophages. For example, at 24 h, between 10⁴ and 10⁵ cfus were recovered for NT1008, 253, 241 and 477. In contrast, only 500 viable organisms remained in the case of isolate NT1542.

Since polymyxin B sulfate may slowly accumulate in macrophages over the 24-h intracellular incubation period, we sought to confirm that the observed differences in intracellular survival were not caused by subtle differences in the sensitivity of each strain to this antibiotic. The minimum inhibitory concentration of polymyxin B sulfate for all strains was determined to be 4 μg ml⁻¹. At 2 μg ml⁻¹, only 13 out of the 33 isolates were killed, indicating differences in the susceptibility of these strains to polymyxin B sulfate (Table 1). These differences, however, did not coincide with the relative ability of each strain to survive in macrophages. For example strains NT401, 699 and 1233, which survived in macrophages, were killed by 2 μg ml⁻¹ of polymyxin B sulfate. In contrast, NT1151, 487, 279 and 1124, which were never recovered from macrophages, were resistant to this concentration of antibiotic. Differences in the number of cfus recovered from macrophages after 24 h also did not appear to reflect subtle differences in sensitivity to polymyxin B sulfate amongst strains. For instance,
we compared the sensitivity of NT723 ($2.1 \times 10^2$ cfus recovered from macrophages after 24 h) and NT1117 ($1.5 \times 10^3$ cfus recovered from macrophages after 24 h) to polymyxin B sulfate. 2.5% of NT723 were resistant to killing by 2 µg ml$^{-1}$ polymyxin B sulfate compared to 0.21% of NT1117. The greater intracellular survival ability of NT1117 therefore did not appear to reflect a lower sensitivity to killing by polymyxin B sulfate accumulated in macrophages.

The observed variation in the ability of NTHi strains to survive in macrophages may therefore reflect the genetic diversity inherent amongst NTHi, and suggests that although the majority of NTHi strains can survive in macrophages, only some strains may have the capacity to persist, for long periods, in this environment.

4. Discussion

Although some earlier reports have suggested NTHi are capable of entering into and surviving in host cells, little work has been carried out to investigate if this is a true

![Fig. 2. Detection of intracellular *H. influenzae* NT477 by immunofluorescence microscopy. Infected monolayers were permeabilized and probed with MAHi 419, an LPS-specific Mab which recognises NT477 LPS. Bound Mab was then detected with a FITC-labelled secondary antibody. Infected monolayers were visualised at (a) 0 h, (b) 2 h, (c) 4 h, (d) 8 h and (e) 24 h following addition of polymyxin B sulfate.](image-url)
occurrence [8,10]. In this study we have shown that many 
nTHi clinical isolates are indeed able to survive in the 
macrophage cell line J774A.1. Furthermore, we have 
shown one strain (NT477) can survive intracellularly for 
several days compared to the nonpathogenic strain Rd 
that is killed within 24 h. The ability of J774 macrophages 
to kill Rd within this time frame provides a measure of the 
bactericidal activity of the cell line used in this study, and 
confirms that the observed survival of NTHi is not a prod-
uct of defective macrophage function.

This ability to survive inside host cells may provide an 
explanation for the common recurrence of NTHi infec-
tions. It is attractive to speculate that during an infection, 
the bacteria would be able to sequester themselves inside 
macrophages present in subepithelial tissue in the respira-
tory tract, and then cause endogenous reinfection at a 
later date. However, whilst we have demonstrated that 
some strains of NTHi have a limited capacity to survive, 
in vitro, in macrophages, our study does not confirm 
whether this phenomenon correlates to in vivo persistence. 
In addition, no information regarding the ability of each 
of the strains used in this study to cause recurrent bouts of

<table>
<thead>
<tr>
<th>NTHi strain</th>
<th>Resistance to killing by 2 mg ml$^{-1}$ polymyxin B sulfate</th>
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+ represents strains resistant to killing by 2 μg ml$^{-1}$ polymyxin B sulfate or which were isolated from macrophages following a 24-h intracellular incubation period.
— represents strains which were sensitive to 2μg ml$^{-1}$ polymyxin B sulfate or which were not recovered from macrophages.
otitis media is available, therefore the clinical significance of this phenomenon remains to be unequivocally established. The ability of Bordetella pertussis to cause acute and chronic respiratory infection in children has also been linked to the ability of this organism to survive in macrophages. B. pertussis is capable of surviving in macrophages in vitro for at least 3 days [12]. In addition, in a separate study, viable B. pertussis were found in bronchoalveolar lavage cells, comprising approximately 80% macrophages, until at least 19 days after infection in a mouse model, suggesting that in this case, in vitro intracellular survival may correlate with in vivo persistence [13].

Most (88%) of the NTHi clinical isolates tested were able to survive intracellularly, but differences were observed in their adhesion/uptake into the macrophage and more significantly in the number of viable intracellular organisms recovered after 24 h. Clearly, even in this small sample, the relative ability of NTHi to survive in macrophage is highly variable. Such differences are consistent with the fact that NTHi are a genetically diverse group of organisms, and it is therefore not surprising to see differences in their interaction with host cells [14]. It is possible that only organisms such as NT477, 1008, 253 and 241, for which greater than 10^4 cfus were recovered after 24 h, will have the capacity to sequester themselves in macrophages for long periods and have the potential to cause recurrent infection. These strains may possess attributes not present in other NTHi strains which enhance their ability to resist macrophage-mediated killing, allowing long-term persistence in these cells.

In order to enter cells, the NTHi first must attach to the cell surface, and several proteins have been shown to be involved in this process. Two surface-exposed high-molecular-mass proteins, HMW1 and HMW2, related to the filamentous haemagglutinin of B. pertussis, have been shown to mediate attachment of NTHi to epithelial cells and macrophage [15,16]. Strains which do not express HMW1 and HMW2 (around 25% of NTHi) have been found to express another protein (Hia) also involved in attachment to host cells [17]. However, another adhesion (Hap) has been shown to promote cell entry when expressed in a noninvasive laboratory strain of H. influenzae. This level of entry is relatively low, however, suggesting that only organisms such as NT477, 1008, 253 and 241, for which greater than 10^4 cfus were recovered after 24 h, will have the capacity to sequester themselves in macrophages for long periods and have the potential to cause recurrent infection. These strains may possess attributes not present in other NTHi strains which enhance their ability to resist macrophage-mediated killing, allowing long-term persistence in these cells.

References


