Journal of General Microbiology (1991), **137**, 1577–1581. *Printed in Great Britain*

Growth of *Haemophilus influenzae* type b in the presence of bovine aortal endothelial cells

P. R. LANGFORD* and E. R. MOXON

Department of Paediatrics, Level 4, John Radcliffe Hospital, Oxford OX3 9DU, UK

(*Received 22 November 1990; revised 8 February 1991; accepted 4 March 1991*)

In serum-free medium in the presence of bovine aortal endothelial cells (BAOEC), *Haemophilus influenzae* type b was capable of extensive proliferation compared to that in serum-free medium alone. An unidentified low-molecular-mass (< 2000 kDa) compound(s) was, in part, responsible for this phenomenon. There were changes in the outer-membrane protein profiles between broth-grown (the original inoculum) and BAOEC-grown organisms, particularly in the 45–70 kDa range. Both broth- and BAOEC-grown bacteria were serum sensitive *in vitro* but could be converted to a serum-resistant phenotype, resembling that found *in vivo*, by incubation in a serum filtrate.

Introduction

Haemophilus influenzae is one of the commonest causes of bacterial meningitis in the UK and USA, with serotype b strains accounting for > 90% of all cases (Turk, 1984). However, the mechanism(s) by which the organism passes from the nasopharynx to the blood and subsequently to the cerebrospinal fluid are unknown. The possibility of *H. influenzae*–endothelial cell interactions occurs when the organisms enter the vascular compartment prior to bloodstream dissemination and at the endothelial cell/central nervous system barrier. In a rat model of *H. influenzae* meningitis, increased permeability to albumin and increased pinocytotic activity in cerebral endothelia were observed (Quagliarello *et al.*, 1986). Studies designed to elucidate the identity and role of *H. influenzae* surface components in endothelial cell interactions were initiated. As a model system bovine aortal endothelial cells (BAOEC) were chosen due to their comparative ease of isolation and culture compared to brain endothelial cells. In the presence of serum there was a rapid increase in cell numbers, growth curves being identical, irrespective of the presence of BAOEC. However, in serum-free medium, in the presence of BAOEC, *H. influenzae* was capable of extensive proliferation compared to that in serum-free medium alone. This latter phenomenon was investigated further and it was found that BAOEC secrete as yet unknown compounds into the medium allowing extended growth of *H. influenzae*.

Abbreviations: BAOEC, bovine aortal endothelial cells; OMP, outer-membrane protein.

Methods

Bacterium and growth conditions. H. influenzae type b strain Eagan (Anderson *et al.*, 1972) was used. It was grown to mid-exponential phase in Brain Heart Infusion (BHI) broth supplemented with 2 μg NAD ml^{-1} and 10 μg haemin ml^{-1} at 37 °C on an orbital incubator (200 r.p.m.). For viable counts, appropriate dilutions were plated onto BHI agar plus Levinthal base and incubated at 37 °C for 18 h. Before addition to BAOEC, strain Eagan was diluted in pre-warmed (37 °C) MEM (Gibco 041-01090) as indicated in Results.

Isolation and cultivation of BAOEC. Adult BAOEC were prepared by collagenase digestion of isolated vessels (Schwartz, 1978), characterized morphologically and by the presence of factor VIII, and used between the 12th and 25th passage (Harlan *et al.*, 1981). BAOEC were grown to confluence at 37 °C in 5% (v/v) carbon dioxide in MEM, 10% (v/v) foetal bovine serum replacement (Ryan Growth Supplement, Miami, Florida) and 5% (v/v) foetal bovine serum (Gibco). For serum-free conditions BAOEC were washed a minimum of six times in MEM and finally replaced in MEM. Growth of BAOEC monolayers for 20 h in the latter conditions was the source of serum-free BAOEC supernatants. These were centrifuged (3000 *g*, 10 min) and the supernatants used directly or stored at −20 °C until required.

Dialysis experiment. Aliquots (2 ml) of MEM ± bacteria (1×10^4 c.f.u. ml^{-1}) were placed in sterile dialysis bags (Sigma, cut-off 2000 kDa), sealed and aseptically transferred to T25s (C3055, NBL, Northumberland, UK) containing 5 ml MEM with confluent BAOEC monolayers ± bacteria. Viable counts of the contents of the dialysis bags and external culture medium were taken after 20 h.

Outer-membrane protein (OMP) profiles. Outer membranes were obtained by Sarkosyl extraction and OMP profiles determined by SDS-

PAGE as described by Allan *et al.* (1987). Gels were silver-stained using the Amersham Kit (RPN.17). Protein concentration was measured by the modification of the method of Bradford (1976) as described by Stoscheck (1990), using bovine serum albumin as the standard.

Serum sensitivity. BHI- and BAOEC-grown orgnisms were washed once in ice-cold PBS and used directly or after incubation in PBSB ± serum filtrate for 30 min at 0 °C. PBSB is PBS, 0·15 mM-calcium chloride, 0·5 mM-magnesium chloride and 0·1% bovine serum albumin. Human serum filtrate (Anderson *et al.*, 1980) was provided by Dr M. Virji of this department. The anti-somatic (non-capsular) bactericidal activity of human sera was assessed by incubating approximately 1×10^4 c.f.u. ml^{-1} of strain Eagan in 25% (v/v) (in PBSB) normal or complement-inactivated (heat-treated at 56 °C for 30 min) adult human serum absorbed with *H. influenzae* type b capsule (PRP) in a final volume of 100 µl. Each 1 ml of serum was absorbed with 30 µg PRP (b-CAPSA I Vaccine, Praxis Biologicals, Rochester, New York, USA) for 30 min at 37 °C followed by 2 h at 4 °C, and small aliquots were stored at −70 °C until required (Shaw *et al.*, 1976). The percentage of bacteria that remained was determined by comparison of viable counts before and after incubation for 30 min at 37 °C.

Results and Discussion

Growth of H. influenzae in the presence and absence of BAOEC

The growth of *H. influenzae* at two different inocula in the presence and absence of BAOEC in serum-free media is shown in Fig. 1. In contrast to MEM alone, bacterial cell numbers in MEM + BAOEC rapidly increased from time zero and eventually reached a plateau level. The concentration of bacteria at the plateau level was different with the two inoculum sizes used. The basis for this phenomenon is unknown. When bacteria were grown in BAOEC supernatants viable counts increased, although there was some variation in numbers between batches (Fig. 1). In order to determine if the compound(s) released by BAOEC was of high or low molecular mass, an *in situ* dialysis experiment was done (Table 1). Bacteria present in infected dialysis bags (IO, I) were capable of growth to approximately two orders of magnitude lower than bacteria in the external medium (IO, O). The concentration of bacteria in the dialysis bag was lower in the experiment where the external medium had also been inoculated (IO) as compared to when bacteria were present only in the dialysis bag (I). Use of the BAOEC-excreted compound(s) by bacteria in the external medium probably limits the amount available for growth in the dialysis bag in this case (IO). The lower level of bacteria in the dialysis bag inoculated alone (I), compared to that found in the external medium (O), may reflect slow diffusion of the compound(s) into the dialysis bag or alternatively that a compound of higher molecular mass may also be involved in the observed phenomenon. This was not pursued further.

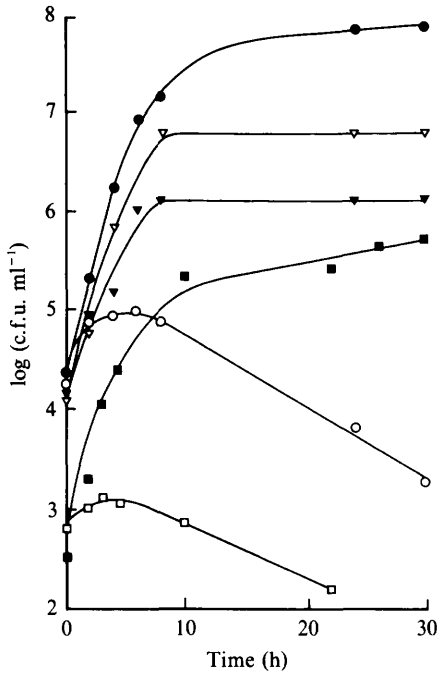

Fig. 1. Growth curves of *H. influenzae*. Starting inoculum of 2×10^4 c.f.u. ml^{-1}; MEM + BAOEC (●); MEM alone (○); two separate batches of BAOEC supernatants (▽, ▼). Starting inoculum of $2{\cdot}9 \times 10^2$ c.f.u. ml^{-1}; MEM + BAOEC (■); MEM alone (□). Results are the means of duplicate samples from duplicate flasks.

The results suggest that *H. influenzae* can use a nutrient(s) derived from BAOEC to sustain growth in the serum-free conditions used in this study. The dialysis experiment indicated that the factor(s) responsible is, at least in part, of low molecular mass (< 2000 kDa). In order to try to identify any compounds that may have been responsible for the observed phenomenon, we have screened amino acids, vitamins, nucleotides, nucleosides and miscellaneous other compounds for their ability to prolong the survival of *H. influenzae* in MEM. The only compounds that individually or combined (final concentration in parentheses), resulted in a marginal increase (< 0·5 log) in bacterial cell numbers at 20 h were: (1) NAD (10 µg ml^{-1}), (2) haemin (10 µg ml^{-1}), (3) glutamine (2 mM) and (4) glutathione (30 µg ml^{-1}). The observed phenomenon is not restricted to BAOEC. High levels (10^5–10^6 c.f.u. ml^{-1}) of *H. influenzae* type b persisted in Eagle's basic medium when grown in the presence of tracheal organ cultures. Histological studies showed that loss of cilia and damage to epithelial cells occurred. A ciliostatic substance, probably lipopolysaccharide (LPS), was released into the growth medium (Denny, 1974).

Table 1. *Dialysis experiment*

Aliquots (2 ml) of MEM ± bacteria (1×10^4 c.f.u. ml^{-1}) were incubated in dialysis bags (cut-off 2000 kDa) placed in 5 ml MEM with confluent BAOEC monolayers ± bacteria (1×10^4 c.f.u. ml^{-1}). Bacterial counts in the dialysis bags and the external medium were determined after 20 h, and are shown ± SEM. The results are the means of duplicate experiments. ND, Below detectable limits.

	Contents of:		Bacterial counts in:	
	Dialysis bag	External medium	Dialysis bag	External medium
IO	MEM + bacteria	MEM + bacteria	$9{\cdot}33 \pm 0{\cdot}36 \times 10^4$	$1{\cdot}33 \pm 0{\cdot}13 \times 10^7$
I	MEM + bacteria	MEM alone	$1{\cdot}97 \pm 0{\cdot}24 \times 10^5$	ND
O	MEM alone	MEM + bacteria	ND	$1{\cdot}23 \pm 0{\cdot}05 \times 10^7$
X	MEM alone	MEM alone	ND	ND

Control monolayers (MEM alone) and those incubated with bacteria maintained their integrity as adjudged morphologically after 8 h. At 20 h there was extensive breakdown of monolayers where bacteria were present; control monolayers were intact (Fig. 2). One possible bacterial substance that could have contributed to detachment and cell death is LPS, an integral component of the Gram-negative cell wall (Lugtenburg & van Alphen, 1983). The cytotoxic effect of either smooth LPS from *Escherichia coli* or rough LPS from *Salmonella minnesota* on BAOEC has been documented (Harlan *et al.*, 1983), BAOEC being less susceptible to *E. coli* LPS when grown under serum-free conditions. *H. influenzae* produces LPS of the rough form (Inzana, 1983). Experiments in this laboratory showed that LPS extracted from strain Eagan was cytotoxic to BAOEC when lactate dehydrogenase release was used as the indicator of cell damage (Langford *et al.*, 1991). In rats, intra-cisternal inoculation of purified *H. influenzae* LPS caused increased blood–brain barrier permeability (Wispelway *et al.*, 1988).

OMP profiles

Bacteria can alter their metabolism and composition in response to environmental change. The expression of surface components *in vivo* can be very different to that *in vitro* (Brown & Williams, 1985). *H. influenzae* type b has the ability to survive in many different growth environments, including the nasopharynx, blood, macrophages (Williams *et al.*, 1991) and cerebrospinal fluid. Notwithstanding the many *in vivo* modes of growth possible, the fact that *H. influenzae* can use a compound(s) for growth from BAOEC suggested that this *in vitro* environment might mimic that *in vivo* more closely than BHI. To determine whether a phenotypic change had occurred in surface components, OMP profiles of BAOEC- and BHI-grown bacteria were determined. BAOEC- and BHI-grown cells were compared since only a few bacteria remained in MEM alone after 20 h and BHI-grown cells were the original inoculum used.

There were more minor proteins in BAOEC-supernatant-grown bacteria, particularly in the range 45–70 kDa (Fig. 3). Similar results were found with two separate extractions. When BAOEC supernatant alone was used no Sarkosyl-insoluble material was found, although a single 48 kDa protein was found when BAOEC were used as the starting material (data not shown). The changes observed may be due to the different growth media used (BHI vs MEM) or different growth phase (exponential with BHI-grown cells and stationary with MEM-supernatant-grown cells). In both strain Eagan in the same range (P. R. Langford, A. Williams & E. R. Moxon, unpublished) and in another *H. influenzae* type b strain in the 50–75 kDa range (van Alphen *et al.*, 1990), minor proteins were more abundant in outer membranes isolated from bacteria harvested directly from the peritoneal cavity of infected rats than in those obtained from broth-grown organisms. It was postulated that, in the latter study, some of these additional proteins were iron-regulated. Morton & Williams (1990) showed that strain Eagan had the same OMP profile, with silver staining, when grown in iron-replete or iron-limited conditions. It is possible, therefore, that the additional proteins found in BAOEC-grown strain Eagan are not iron regulated.

Serum sensitivity

Broth-grown strain Eagan is more susceptible to the complement-mediated bactericidal activity of anti-somatic antibodies than are organisms harvested directly from the nasopharynx (Rubin & Moxon, 1985) or the blood of infected rats (Shaw *et al.*, 1976). Broth-grown serum-sensitive strain Eagan can be converted to the serum-resistant phenotype, resembling that observed *in*

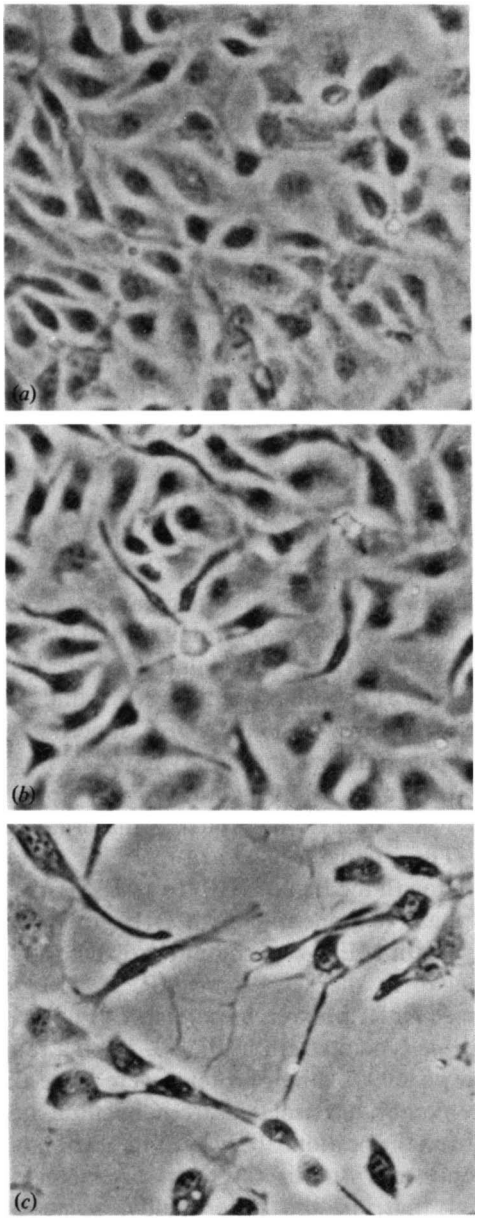

Fig. 2. Phase-contrast photographs of confluent BAOEC monolayers grown for 20 h in (*a*) MEM, 10% foetal bovine serum replacement and 5% foetal bovine serum; (*b*) MEM alone; and (*c*) MEM + *H. influenzae* (starting inoculum 1×10^4 c.f.u. ml^{-1}).

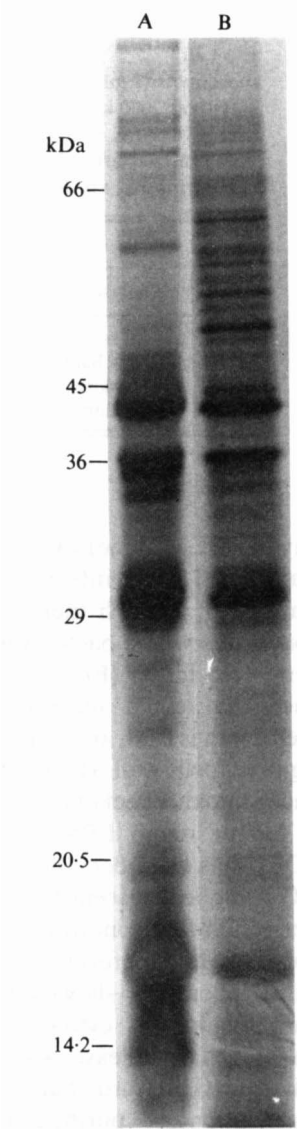

Fig. 3. SDS-PAGE gel (12·5%, w/v, acrylamide; 10 μg protein per lane) of (A) BHI-grown and (B) BAOEC-supernatant-grown *H. influenzae*.

vivo, by incubation in filtrates of rat (Shaw *et al.*, 1976) or human (Anderson *et al.*, 1980) sera. The anti-somatic bactericidal activities of human sera against BHI- and BAOEC-grown orgnisms were therefore compared. There was a reproducible statistically significant ($P < 0{\cdot}01$, Student's t-test), but not substantial, difference in the mean number of bacteria that remained between samples which had been incubated in normal (PRP-absorbed) serum. Both BHI- and BAOEC-grown organisms were converted to the serum-resistant phenotype after incubation in serum filtrate (Table 2). Use of BHI- or BAOEC-grown organisms directly, rather than pre-incubated in PBSB for 30 min at 0 °C prior to the bactericidal assay, made no difference to the results (data not shown). Diminished bactericidal activity was observed for heat-treated (complement-inactivated) sera in all cases. Complement is crucial for the normal clearance of *H. influenzae* from blood (Crosson *et al.*, 1976), the most likely mechanism being C3 mediating association of organisms with macrophages (Noel *et al.*, 1990).

As knowledge accumulates of the composition and characteristics of *in vivo*-grown organisms then it should

Table 2. *Sensitivity of strain Eagan to normal and heat-treated (complement-inactivated) adult human sera absorbed with PRP*

The percentages of bacteria ± SEM that remained after incubation of 1×10^4 c.f.u. ml^{-1} bacteria in serum for 30 min at 37 °C are shown. BHI- and BAOEC-grown bacteria were pre-incubated in PBSB or serum filtrate for 30 min at 0 °C prior to the bactericidal assay. The results are the means of triplicate determinations.

Growth condition + pre-incubation medium	Human serum (PRP absorbed)	
	Normal	Heat treated
BHI ± PBSB	0·78 ± 1·08	125·21 ± 18·95
BHI + filtrate	106·58 ± 8·18	135·00 ± 8·75
BAOEC + PBSB	5·97 ± 3·25	111·66 ± 18·37
BAOEC + filtrate	100·90 ± 6·62	96·47 ± 11·52

be possible to formulate media and growth conditions *in vitro* which mimic more closely those found *in vivo*. Whilst BAOEC-grown organisms did not possess the serum resistance trait associated with *in vivo*-grown bacteria, it remains to be determined whether the additional OMPs found are present in infection and have a role in pathogenesis.

This work was supported by MRC Programme and Wellcome Trust grants to E. R. M. Thanks are due to Dr A. Hussein for conveying the original observation and for helpful discussions.

References

ALLAN, I., LOEB, M. R. & MOXON, E. R. (1987). Limited genetic diversity of *Haemophilus influenzae* (type b). *Microbial Pathogenesis* **2**, 139–145.

VAN ALPHEN, L., GEELEN-VAN DEN BROEK, L. & VAN HAM, M. (1990). *In vivo* and *in vitro* expression of outer membrane components of *Haemophilus influenzae*. *Microbial Pathogenesis* **8**, 279–288.

ANDERSON, P., JOHNSTON, R. B. & SMITH, D. H. (1972). Human serum activities against *Haemophilus influenzae*, type b. *Journal of Clinical Investigation* **51**, 31–38.

ANDERSON, P., FLESHER, A., SHAW, S. & HARDING, A. L. (1980). Phenotypic and genetic variation in the susceptibility of *Haemophilus influenzae* type b to antibodies to somatic antigens. *Journal of Clinical Investigation* **65**, 885–891.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of dye binding. *Analytical Biochemistry* **72**, 248–254.

BROWN, M. R. W. & WILLIAMS, P. (1985). The influence of the environment on envelope properties affecting the survival of bacteria in infection. *Annual Review of Microbiology* **39**, 527–556.

CROSSON, F. J., WINKELSTEIN, J. A. & MOXON, E. R. (1976). Participation of complement in the non-immune host defense against experimental *Haemophilus influenzae* type b septicemia and meningitis. *Infection and Immunity* **14**, 882–887.

DENNY, F. W. (1974). Effect of a toxin produced by *Haemophilus influenzae* on cultured respiratory epithelium. *Journal of Infectious Diseases* **129**, 93–100.

HARLAN, J. M., KILLEN, P. D., HARKER, L. A., STRIKER, G. E. & WRIGHT, D. G. (1981). Neutrophil-mediated endothelial injury *in vitro*: mechanisms of cell detachment. *Journal of Clinical Investigation* **68**, 1394–1403.

HARLAN, J. M., HARKER, L. A., REIDY, M. A., GAJDUSEK, C. M., SCHWARTZ, S. M. & STRIKER, G. E. (1983). Lipopolysaccharide-mediated bovine cell injury *in vitro*. *Laboratory Investigation* **48**, 269–274.

INZANA, T. J. (1983). Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of *Haemophilus influenzae*. *Journal of Infectious Diseases* **148**, 492–499.

LANGFORD, P. R., SZABO, M. & MOXON, E. R. (1991). *In vitro* cytotoxicity of *Haemophilus influenzae* lipopolysaccharide for bovine aortal endothelial cells. *FEMS Microbiology Letters* (in the press).

LUGTENBURG, B. & VAN ALPHEN, L. (1983). Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacteria. *Biochimica et Biophysica Acta* **737**, 51–115.

MORTON, D. J., & WILLIAMS, P. (1990). Siderophore-independent acquisition of transferrin bound iron by *Haemophilus influenzae* type b. *Journal of General Microbiology* **136**, 927–934.

NOEL, G. J., MOSSER, D. M. & EDELSON, P. J. (1990). Role of complement in mouse macrophage binding of *Haemophilus influenzae* type b. *Journal of Clinical Investigation* **85**, 208–218.

QUAGLIARELLO, V. T., LONG, W. J. & SCHELD, W. M. (1986). Morphological alterations of the blood–brain barrier with experimental meningitis in the rat. Temporal sequence and role of encapsulation. *Journal of Clinical Investigation* **77**, 1084–1095.

RUBIN, L. G. & MOXON, E. R. (1985). The effect of serum factor induced resistance to somatic antibodies on the virulence of *Haemophilus influenzae* type b. *Journal of General Microbiology* **131**, 515–520.

SCHWARTZ, S. M. (1978). Selection and characterization of bovine aortic endothelial cells. *In Vitro* **14**, 966–980.

SHAW, S., SMITH, A. L., ANDERSON, P. & SMITH, D. H. (1976). The paradox of *Haemophilus influenzae* type b bacteremia in the presence of serum bactericidal activity. *Journal of Clinical Investigation* **58**, 1019–1029.

STOSCHECK, C. M. (1990). Increased uniformity in the response of the coomassie blue G protein assay to different proteins. *Analytical Biochemistry* **184**, 111–116.

TURK, D. C. (1984). The pathogenicity of *Haemophilus influenzae*. *Journal of Medical Microbiology* **18**, 1–16.

WILLIAMS, A., MASKELL, D. & MOXON, E. R. (1991). Relationship between intracellular survival in macrophages and virulence of *Haemophilus influenzae* type b. *Journal of Infectious Diseases* (in the Press).

WISPELWAY, B., LESSE, A. J., HANSEN, E. J. & SCHELD, W. M. (1988). *Haemophilus influenzae* lipopolysaccharide induced blood–brain barrier permeability during experimental meningitis in the rat. *Journal of Clinical Investigation* **82**, 1339–1346.