The Effect of Serum-factor Induced Resistance to Somatic Antibodies on the Virulence of *Haemophilus influenzae* Type b

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Studies on the pathogenesis of *Haemophilus influenzae* b infection have used bacteria grown *in vitro* which are relatively serum-sensitive (using serum devoid of anticapsular antibody) compared to organisms taken from infected hosts. We compared the virulence of relatively serum-sensitive and serum-factor induced serum-resistant *H. influenzae* b by inoculating rats with organisms having one or the other phenotype. The serum-resistant phenotype was more virulent following intraperitoneal or intravenous inoculation; however, there was no difference in the incidence of colonization or bacteraemia following intranasal inoculation. Furthermore, organisms colonizing the pharynx of rats had the serum-resistant phenotype. Thus, different phenotypes of the same strain of *H. influenzae* b differed in virulence following parenteral, but not intranasal, inoculation of bacteria. This could be explained by a change from serum-sensitive to serum-resistant phenotype shortly after entering the nasopharynx. The phenotype of micro-organisms grown *in vitro* may differ from organisms in infected individuals and these differences may be of critical importance in studies of immunity to infection and the pathogenesis of infection.

INTRODUCTION

Studies on the virulence of the human pathogen *Haemophilus influenzae* have been facilitated by the availability of a biologically relevant rat model. In studying virulence, most investigators inoculate suspensions of organisms which are grown *in vitro*. During *in vitro* growth, a shift in the phenotype of the organisms may occur (Shaw *et al.*, 1976; Ward *et al.*, 1970). A striking example of phenotypic change has been described for *H. influenzae* type b where organisms from infected rats are relatively resistant to bactericidal activity involving antibodies to somatic antigens compared to the identical strain grown in synthetic media (Shaw *et al.*, 1976). Since broth-grown, serum-sensitive organisms become relatively serum-resistant following 30 min incubation in normal serum, it is evident that this resistance reflects a phenotypic shift rather than selection of genetically distinct, serum-resistant organisms. Anderson *et al.* (1980) showed that this relative serum resistance involves an altered interaction of serum antibodies with lipopolysaccharide (LPS) determinants; both phenotypes are sensitive to bactericidal activity mediated by anticapsular antibodies. A potential biological relevance of these observations was suggested since limited experiments showed that intravascular clearance of broth-grown, serum-sensitive *H. influenzae* b was greater than that of the same strain which was pre-incubated in serum to induce resistance to somatic antibodies (Shaw *et al.*, 1976). Thus, as previous studies of *H. influenzae* b pathogenesis using the rat model have used broth-grown organisms (Smith *et al.*, 1973; Moxon *et al.*, 1974; Weller *et al.*, 1977; Rubin & Moxon, 1983; Moxon & Vaughn 1981),

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these studies may not have accurately simulated the pathogenesis observed following natural infection. In the present studies, we incubated broth-grown serum-sensitive *H. influenzae* b in a dialysate of serum to obtain serum-resistant organisms and we compared the virulence of the serum-sensitive and serum-resistant phenotypes of *H. influenzae* b following systemic and intranasal inoculation of rats.

METHODS

Organisms, media and growth. One-step streptomycin-resistant (Sm^r), kasugamycin-resistant (Kg^r), or rifampin-resistant (Ri^r) mutants of *H. influenzae* b strain Eag were used (Smith *et al.*, 1973). Strain S-2 is a spontaneous capsule-deficient mutant of strain Eagan Sm^r (Weller *et al.*, 1977). Strain Rd is a capsule-deficient *H. influenzae* strain (Alexander & Leidy, 1951). Broth-grown *H. influenzae* b were grown and prepared for animal inoculation as previously described (Rubin & Moxon, 1983). For quantification of colony counts, body fluids and appropriate dilutions of the inoculum were cultured on solid media as previously described (Rubin & Moxon, 1983), with added streptomycin (500 µg ml⁻¹), kasugamycin (100 µg ml⁻¹), or rifampin (100 µg ml⁻¹).

Serum-resistant phenotype, bactericidal assay. Broth-grown, mid-exponential phase cultures of *H. influenzae* b were pelleted, resuspended in buffer or in a low molecular weight dialysate of normal human serum to a bacterial density of 10⁴ organisms ml⁻¹, and incubated for 30 min at 37 °C to obtain bacteria with the serum-sensitive or serum-resistant phenotype, respectively (Anderson *et al.*, 1980). Serum resistance was assessed in a serum bactericidal assay. Organisms (200 µl) were incubated at 37 °C in 20 % immune serum (50 µl). (The immune serum, containing a high proportion of anti-LPS antibodies but lacking anti-capsular antibody, was obtained from rats previously inoculated with *H. influenzae* strain Rd and stored at -70 °C to preserve complement activity.) Samples (10 µl) were quantitatively cultured initially and following a 20 or 30 min incubation. There was generally either a <10% or >70% decrease in the colony counts following incubation. Organisms were considered serum-sensitive if there was a >50% decline in colony counts during the 20 or 30 min incubation. Serum was defined as having bactericidal activity if there was a >50% decline in viable counts of broth-grown *H. influenzae* b following a 20 min incubation.

Animals. Sprague-Dawley rats strain COBS (obtained from Charles River Laboratories, Wilmington, Mass., USA) were used in all experiments.

Intravenous inoculation studies. To induce serum antibodies to LPS, 5-d-old rats were inoculated intranasally with 10⁷ capsule-deficient *H. influenzae* strain S-2. Bactericidal activity of serum was confirmed using serum taken at 38 d. At 40 d old these rats were inoculated intravenously with approximately 1000 serum-resistant or serum-sensitive *H. influenzae* b via a lateral tail vein after light ether anaesthesia. Rats of this age are highly susceptible to bacteraemia following intravenous inoculation with *H. influenzae* b. At varying intervals after inoculation, a single blood sample was obtained by heart puncture from groups of three or four animals and quantitatively cultured on solid media (limit of detection 10 organisms ml⁻¹).

Intraperitoneal inoculation studies. Unanaesthetized 14-d-old rats were inoculated intraperitoneally with 10⁴ organisms of either the serum-sensitive or serum-resistant phenotype. Blood (10 µl) was obtained for culture following tail vein puncture at 24 h. Animals were observed for death occurring within 7 d of inoculation.

Intranasal inoculation studies. Rats 20 d old, previously shown to be highly susceptible to *H. influenzae* b colonization and bacteraemia following intranasal inoculation (Rubin & Moxon, 1983), were housed in separate cages to avoid intralitter spread of *H. influenzae* b (Halsey *et al.*, 1980) and were intranasally inoculated (25 µl sample containing varying numbers of organisms) as previously described (Moxon & Vaughn, 1981). Pharyngeal secretions were obtained for culture or as a source of organisms for the bactericidal assay as previously described (Rubin & Moxon, 1983). Blood was obtained for culture (25 µl) or use in a bactericidal assay following tail vein puncture.

RESULTS

Bacteraemia following intravenous inoculation of serum-sensitive or serum-resistant H. influenzae b

Blood cultures were obtained 5 min to 24 h following intravenous inoculation with serum-sensitive or serum-resistant *H. influenzae* b. Combining all time points sampled, there was a higher magnitude of bacteraemia in rats inoculated with serum-resistant organisms than in those inoculated with serum-sensitive *H. influenzae* b ($P < 0.05$, Wilcoxon 2-sample rank sum test, 2-tailed) (Fig 1). At 1 and 3 h after inoculation, there was a significantly higher magnitude of bacteraemia in rats inoculated with serum-resistant *H. influenzae* b than in those inoculated with serum-sensitive organisms [95% confidence intervals of log₁₀ geometric mean bacteria per ml blood: (2.71, 2.99) vs (1.06, 2.10) at 1 h and (3.15, 3.55) vs (1.72, 2.36) at 3 h].

Fig. 1. Bacteraemia following intravenous inoculation of 40-d-old rats with 10^3 serum-sensitive (○) or serum-resistant (●) *H. influenzae* b. Each point represents the geometric mean magnitude of bacteraemia for three or four rats; blood was obtained for culture once for each rat. Bars represent one SD of the mean. Rats had been intranasally inoculated at 5 d of age with 10^7 *H. influenzae* b to ensure the presence of serum bactericidal antibodies to LPS.

Bacteraemia and mortality following intraperitoneal inoculation of serum-sensitive or serum-resistant H. influenzae b

To compare further the virulence of the serum-sensitive and serum-resistant phenotypes of *H. influenzae* b, 14-d-old rats were inoculated intraperitoneally with 10^4 organisms of one or the other phenotype. All animals developed bacteraemia; there was a significantly greater mortality after inoculation with the serum-resistant phenotype (16/19) than after inoculation with the serum-sensitive phenotype (8/18) ($P < 0.05$, χ^2). Thus, a difference in virulence of the two phenotypes could be demonstrated following systemic administration of organisms.

Phenotype of H. influenzae b colonizing the pharynx

Since infection in humans is initiated in the nasopharynx, the relevance of these observed differences in virulence following systemic inoculation of organisms was uncertain. Therefore, we studied the phenotype of organisms colonizing the nasopharynx and compared colonization and bacteraemia following intranasal inoculation with the serum-sensitive or serum-resistant phenotype of *H. influenzae* b.

Pharyngeal secretions were obtained from nine colonized rats (from six different litters) 5 to 7 d after intranasal inoculation with approximately 10^4 *H. influenzae* b at age 20 d. Serial 10-fold dilutions of these secretions were assayed in a serum bactericidal assay (see Methods). *H. influenzae* b in secretions survived the 30 min incubation in comparison to the survival of broth-grown organisms (Table 1). Thus, *H. influenzae* b colonizing the pharynx were of the serum-resistant phenotype. Furthermore, when broth-grown organisms were pre-incubated with filter sterilized pharyngeal washes, there was a higher percentage survival in the bactericidal assay (mean 19%, $n = 5$) as compared to organisms pre-incubated with buffer (percentage survival 2%). Although the mean percentage survival was lower than that for bacteria which had been pre-incubated with dialysate (117%), this nevertheless indicated that a factor(s) necessary for induction of serum resistance is present in pharyngeal secretions.

Colonization efficiency of serum-sensitive and serum-resistant phenotypes of H. influenzae b

To maximize the chances of detecting differences in colonization efficiency between the two phenotypes, an inoculum size representing the ID_{50} for colonization (10^2 – 10^3 organisms) was used. There was no difference in the incidence of colonization following intranasal inoculation with either the serum-sensitive or serum-resistant phenotype of *H. influenzae* b (Table 2a).

Table 1. *Survival of H. influenzae b from pharyngeal washes and broth-grown culture in a serum bactericidal test*

20-d-old rats were inoculated intranasally with approximately 10^4 *H. influenzae b*; pharyngeal washes were obtained 5 to 6 d later. Organisms from both sources were tested in a serum bactericidal assay (see text). (Percentage survival of greater than 100 indicates a net replication of organisms during the 20 or 30 min incubation).

Source of <i>H. influenzae b</i>	Mean percentage survival
Expt no. 1 Pharyngeal washes ($n = 5$)	103
Broth-grown culture ($n = 2$)	21
Expt no. 2 Pharyngeal washes ($n = 4$)	73
Broth-grown culture ($n = 1$)	0
Broth-grown incubated with dialysate ($n = 1$)	113

Table 2. *Nasopharyngeal colonization after intranasal inoculation with serum-sensitive and/or serum-resistant H. influenzae b*

20-d-old rats were inoculated intranasally with approximately 10^3 organisms; colonization was assessed by obtaining pharyngeal washes after 48–72 h which were cultured on solidified media made selective with the appropriate antibiotic. (a) Incidence of colonization following intranasal inoculation of rats with either serum-sensitive or serum-resistant *H. influenzae b*. Data represent combined results of two separate experiments. (b) Incidence of colonization following intranasal inoculation of rats with an approximately equal mixture of serum-sensitive and serum-resistant *H. influenzae b*. These two phenotypes were distinguished by using two different antibiotic-resistant variants of the same *H. influenzae b* strain which had similar colonization efficiency. Data represent combined results of three separate experiments in which a total of 40 rats were inoculated.

	No. colonized/ no. inoculated*
(a) Phenotype inoculated	
Serum-sensitive	10/35 (31)
Serum-resistant	11/35 (29)
(b) Phenotype recovered	
Either	30/40 (75)
Serum-sensitive	15/40 (38)†
Serum-resistant	19/40 (48)†

* Percentages given in parentheses.

† $P > 0.25$ (χ^2).

To compare further the colonization efficiency, individual rats were inoculated with a mixture of serum-sensitive and serum-resistant *H. influenzae b* (with two different antibiotic-resistant markers – Sm^r or Ri^r, see Methods) allowing the serum-sensitive and serum-resistant phenotypes to compete within the same animal, thereby allowing each animal to serve as its own control. There was no significant difference in the incidence of colonization after inoculation with the serum-sensitive and serum-resistant phenotypes of *H. influenzae b* (Table 2b). Parallel experiments compared the colonization efficiency of the two antibiotic-resistant variants of *H. influenzae b* by inoculating rats with a mixture of Sm^r and Ri^r variants of serum-sensitive *H. influenzae b*. The two antibiotic-resistant variants colonized the nasopharynx of rats with approximately equal efficiency (L. G. Rubin & E. R. Moxon, unpublished observation) and thus differences in colonization between these two antibiotic-resistant variants were unlikely to confound the interpretation of this experiment. Thus, serum sensitivity does not appear to be a critical determinant of initial pharyngeal survival and subsequent pharyngeal colonization.

Bacteraemia after intranasal inoculation with serum-resistant or serum-sensitive H. influenzae b

To maximize the chances of detecting differences in the incidence or magnitude of bacteraemia following intranasal inoculation, an inoculum (10^3 – 10^4 organisms) was chosen which would result in a 50–100% incidence of bacteraemia. In order to maximize the chances of detecting differences between the two phenotypes, only rats whose serum (obtained 2 d before

Table 3. *Bacteraemia after intranasal inoculation with serum-sensitive and serum-resistant H. influenzae b*

The incidence and magnitude of bacteraemia after intranasal inoculation of 20-d-old rats with an approximately equal mixture (approximately 3000 organisms) of serum-sensitive and serum-resistant *H. influenzae* b was measured. The two phenotypes were distinguished by using two different antibiotic-resistant variants of the same *H. influenzae* b strain which were of similar virulence. Blood (25 µl) was obtained 72 h after inoculation and was cultured on solidified media made selective with the appropriate antibiotic. The 95% confidence intervals are given in parentheses.

Phenotype recovered	No. bacteraemia/ no. inoculated	Geometric mean bacteria per ml blood
Either	14/18	—
Serum-sensitive	11/18	2.48 (1.95, 3.01)
Serum-resistant	11/18	2.36 (1.88, 2.84)

inoculation) had bactericidal activity against *H. influenzae* b (see Methods) were used in this experiment. Following inoculation with an approximately equal mixture of serum-sensitive and serum-resistant *H. influenzae* b (using Sm^r and Kg^r variants for antibiotic-resistant markers), there was no significant difference in the incidence or magnitude of bacteraemia after inoculation of organisms originally of the serum-sensitive or serum-resistant phenotype (Table 3). Parallel experiments compared the bacteraemia following intranasal inoculation with the Sm^r and Kg^r variants of serum-sensitive *H. influenzae* b. The incidence and magnitude of bacteraemia with these two antibiotic-resistant mutants, possessing similar virulence properties, were similar (L. G. Rubin & E. R. Moxon, unpublished observation). Thus, inoculation with either the serum-sensitive or serum-resistant phenotype of *H. influenzae* b resulted in a similar incidence and magnitude of bacteraemia.

DISCUSSION

The serum-resistant phenotype was more virulent following intravenous or intraperitoneal inoculation than the serum-sensitive phenotype. However, since infection is initiated via the nasopharynx, the biological relevance of these observations to infection was uncertain. Following intranasal challenge with an inoculum containing the ID₅₀ for colonization or bacteraemia, there was no difference between the two phenotypes in the incidence of colonization or bacteraemia, respectively. Furthermore, we showed that organisms colonizing the pharynx of rats have the serum-resistant phenotype. The factor(s) necessary for expression of the serum-resistant phenotype must be present in the nasopharynx as organisms must have acquired this phenotype in the nasopharyngeal environment. If this phenotypic shift had occurred shortly after inoculation, thereby rapidly eradicating any differences in serum-sensitivity between the two inocula, this would provide an explanation for the similar colonization and bacteraemia observed after inoculation with the two phenotypes. The efficient colonization observed following inoculation with a small number of serum-sensitive organisms implies that bactericidal activity involving anti-LPS antibodies may not be an important host defence against *H. influenzae* b colonization and/or organisms inoculated into the nasal cavity acquire serum resistance very shortly after inoculation.

Only one strain of *H. influenzae* b was used in these studies. Strain Eag was chosen because it has been used in many of the experimental studies of *H. influenzae* pathogenesis (Smith *et al.*, 1973; Moxon *et al.*, 1974; Rubin & Moxon, 1983; Alexander & Leidy, 1951). However, the results may be applicable to most type b strains as Anderson *et al.* (1980) found that all eight type b strains tested underwent a phenotypic shift to relative serum resistance. The age of rats chosen for the study of comparative virulence varied with the route of inoculation (i.e. 40-d-old rats for intravenous inoculation, 14-d-old rats for intraperitoneal inoculation, 20-d-old rats for intranasal inoculation). These particular ages were chosen for the reproducibility with which rats could be infected by the particular route. However, since intravenous or intraperitoneal inoculation results in bacteraemia in rats aged 14, 20 or 40 d and intranasal inoculation results in colonization and bacteraemia in rats aged 14 or 20 d, it is unlikely that the use of rats of other ages would have altered the results.

Differences in serum sensitivity between organisms from infected hosts and organisms grown *in vitro* have also been demonstrated for *Neisseria gonorrhoeae*. Although strains from disseminated infection may retain their resistance to serum on subculture and thus represent genetically stable, serum-resistant organisms, isolates from mucosal sites may lose their resistance upon subculture and regain phenotypic resistance to killing by human serum following incubation in genital secretions (Martin *et al.*, 1982). Thus, *in vitro*-grown *N. gonorrhoeae* may undergo a phenotypic shift to relative serum resistance similar to *H. influenzae* (Ward *et al.*, 1970). Although urethral exudates from infected individuals tend to infect volunteers more readily than *in vitro*-grown organisms (Hill, 1943), the lack of a biologically relevant animal model for this organism has hampered attempts to compare the virulence of the serum-sensitive and serum-resistant phenotypes in a controlled fashion.

In studying immunity to bacterial infections and the pathogenesis of bacterial infections, the phenotype of the organisms may be of critical importance to virulence and the phenotype of organisms grown on artificial media may differ in important ways from the phenotype of the organisms in the infected host.

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