INFECTION AND IMMUNITY, Nov. 1997, p. 4701–4704 Vol. 65, No. 11
0019-9567/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Development of a Model of Low-Inoculum *Streptococcus pneumoniae* Intrapulmonary Infection in Infant Rats

RICHARD A. SALADINO,[1]* ANNE M. STACK,[1] GARY R. FLEISHER,[1] CLAUDETTE M. THOMPSON,[2] DAVID E. BRILES,[3] LES KOBZIK,[4] AND GEORGE R. SIBER[2]

Department of Medicine, Children's Hospital,[1] and Laboratory of Infectious Diseases, Dana-Farber Cancer Institute,[2] Harvard Medical School, and Department of Pathology, Brigham and Women's Hospital,[4] Boston, Massachusetts, and Department of Microbiology, University of Alabama, Birmingham, Alabama[3]

Received 5 May 1997/Returned for modification 10 June 1997/Accepted 8 August 1997

We have developed a model of low-inoculum *Streptococcus pneumoniae* infection in infant rats. We challenged 4-day-old Sprague-Dawley pups via intraperitoneal or intrapulmonary injection of *S. pneumoniae* serotypes 1, 3, 4, 5, 6b, 7f, 9v, 14, 19f, and 23f. To achieve bacteremia with low inocula, it was necessary to passage the isolates in rats. Inocula of the 10 *S. pneumoniae* serotypes producing bacteremia in 50% or more animals ranged from 1 to 400 CFU. Virulence was similar by intraperitoneal and intrapulmonary routes. Lung specimens from animals challenged by the intrapulmonary route grew *S. pneumoniae* and demonstrated histologic evidence of focal infection. Meningitis was detected in 20 to 50% of bacteremic animals, and mortality invariably followed bacteremia within 24 to 48 h. This model of intrapulmonary infection uses low inocula of *S. pneumoniae* and results in bacteremia, meningitis, and death in infant rats.

Invasive *Streptococcus pneumoniae* infection is a worldwide problem. The pneumococcus is the most common cause of bacterial pneumonia (3, 7, 19, 20) and is also an important cause of otitis media, bacteremia, and meningitis (4, 15). In response to increasing rates of penicillin-resistant pneumococci, efforts to reduce associated morbidity and mortality have focused on the development of more efficacious antimicrobials (6, 10, 11) and vaccines that are immunogenic in infants and young children (8, 13, 16, 18).

Current animal models of *S. pneumoniae* infection are limited. The higher-numbered pneumococcal serotypes that commonly cause pediatric infection (e.g., serotypes 9, 14, 18, 19, and 23) are not reliably pathogenic for mice. Also, existing intraperitoneal models of infection do not mimic human pathogenesis and require challenge doses of pneumococci that are very high (e.g., 10^3 to 10^7 organisms) (1, 2, 12, 14, 17). We sought to develop in infant rats a pulmonary infection model of *S. pneumoniae* which would more closely mimic human disease and allow for the evaluation of passive immunization against infection with *S. pneumoniae*. In particular, our objectives for this model included the production of pneumococcal infection from low-challenge inocula of pneumococcal strains prevalent in children in developed and underdeveloped countries, the presence of focal (intrapulmonary) infection, and evidence of invasive pneumococcal infection, including bacteremia, meningitis, and death.

MATERIALS AND METHODS

Bacteriologic methods. Several strains of *S. pneumoniae*, representing serotypes 1, 3, 4, 5, 6b, 7f, 9v, 14, 19f, and 23f, were obtained from the collections of George Siber (Dana-Farber Cancer Institute, Boston, Mass.), Gerald Schiffman (State University of New York, Brooklyn), and David Briles (University of Alabama, Birmingham) (Table 1). Pneumococci were identified by optochin sensitivity and typed by quellung capsular swelling reaction. After animal passage, pneumococcal type and subtype were confirmed in the laboratory of Richard Facklam (Centers for Disease Control, Atlanta, Ga.).

Strains were stored in either skim milk or Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) and 20% glycerol at −70°C, and fresh subcultures were used for all experiments. Inocula for animal challenge were prepared by growing *S. pneumoniae* to mid-log phase (approximately 10^7 CFU/ml) in 10 ml of Todd-Hewitt broth supplemented with 0.5% yeast extract. The suspension was diluted in 0.5% low-melting-point agarose (as an adjuvant) to a desired inoculum concentration and stored on wet ice until the intraperitoneal or intrathoracic inoculation. The actual inoculum CFU was calculated based on the dilutions made from the mid-log-phase culture.

To assess lung microbiology, 1 day after *S. pneumoniae* intrapulmonary challenge, lungs were dissected en bloc, in sterile fashion, from the thorax, transported in sterile glass vials, weighed, and then homogenized in a Tissue Tearor (Biospec Products, Inc., Bartlesville, Okla.). Lung specimens were also obtained for histologic examination; lungs were preserved via tracheal instillation of 10% formalin immediately upon dissection.

Cultures of blood and cerebrospinal fluid were performed on 5% sheep blood agar. Cultures of lung homogenate were performed on 5% sheep blood agar supplemented with gentamicin to inhibit the growth of normal flora. Plates were incubated overnight at 37°C, and colonies were counted the following morning.

Animal model. Outbred virus-free Sprague-Dawley rats were obtained from Charles River Laboratories, Wilmington, Mass.). Pregnant female rats were

TABLE 1. *S. pneumoniae* strains used and inocula producing 50 to 90% bacteremia

Serotype	Strain[a]	Inoculum (CFU/rat) producing 50 to 90% bacteremia	
		Before passage	After passage
1	L8-2006.RS	2×10^2	1
3	GSc-3.RS	2×10^2	0.5
4	BG-7395.RS	1×10^4	4
5	DBL-3.RS	2×10^5	5
6b	GSc-6B.RS	2×10^7	5
7f	GSc-7F.RS	1×10^4	400
9v	AC-107.RS	1×10^6	20
14	EF-16565.RS	3×10^3	5
19f	L8-2013.RS	2×10^5	50
23f	D109-18.RS	3×10^3	1

[a] Serotype 1, 4, 5, 9v, 14, 19f, and 23f originated from the laboratory of D. E. Briles (University of Alabama, Birmingham); the other strains originated from the laboratory of G. R. Siber (Dana-Farber Cancer Institute, Boston, Mass.) (original gifts of G. Schiffman [State University of New York, Brooklyn]). "RS" indicates that the strain was animal passaged in our laboratory.

* Corresponding author. Mailing address: Children's Hospital, 300 Longwood Ave., Boston, MA 02115. Phone: (617) 355-6624. Fax: (617) 355-6625. E-mail: saladino@a1.tch.harvard.edu.

TABLE 2. Day 7 mortality rates after challenge with *S. pneumoniae* types 6b and 23f

Route	Day 7 mortality rate (no. dead/group of 8)					
	Serotype 6b inoculum (CFU/rat)			Serotype 23f inoculum (CFU/rat)		
	0.01	0.1	1.0	0.2	2.0	20
Intraperitoneal	0	2	4	2	4	7
Intrapulmonary	0	2	3	2	7	8

allowed to deliver in our animal housing units. On day 4 of life, infant pups from all litters were randomly redistributed so that each mother had 10 to 12 pups.

Intraperitoneal inoculation with *S. pneumoniae* was performed to passage the bacteria in an attempt to increase virulence. The abdomen of each 4-day-old pup was prepared with 70% isopropyl alcohol, and a 0.10-ml inoculum in agarose was injected intraperitoneally via a 27-gauge needle.

Intrathoracic inoculations were performed in the following fashion. The right chest of each 4-day-old pup was prepared with alcohol, and a 0.025-ml inoculum was injected transthoracically into the mid-right lung via a 29-gauge needle on an insulin syringe. The depth of injection was controlled by a small hemostat clipped 3 mm from the needle bevel.

Bacteremia was assessed 1, 2, and 3 days after inoculation. The distal dorsal tail vein of each unanesthetized pup was cleansed with 70% alcohol and punctured with a sterile lancet, and 0.01 ml of blood was spread on 5% sheep's blood agar. The presence or absence of growth of *S. pneumoniae* was recorded for each day after incubation. Cerebrospinal cultures were obtained 1, 2, and 3 days after inoculation. Pups were held with the head flexed, the occipital area was cleansed with 70% alcohol, and puncture of the cisterna magna was performed with a 27-gauge needle. Approximately 0.10 ml of cerebrospinal fluid was spread on plates containing supplemented brain heart infusion agar. The presence or absence of growth of *S. pneumoniae* was recorded for each day after incubation. During the inoculation and culture procedures, pups were kept warm under a 40-W light source. Mortality was assessed for 7 days after inoculation. Experimental procedures for use with animals were reviewed and approved by the Children's Hospital Animal Care and Use Committee and were in keeping with the guidelines of the National Institutes of Health.

RESULTS

Animal passage of bacterial isolates. Recovery of an isolate of *S. pneumoniae* from a rat pup bacteremic after intraperitoneal inoculation was considered animal passage. Passage of an isolate after intraperitoneal inoculation uniformly resulted in increased virulence (Table 1). Before passage, inocula required to produce 50 to 90% bacteremia ranged from 10^3 to 10^7 organisms. After animal passage, inocula required to produce 50 to 90% bacteremia ranged from 1 to 400 organisms for serotypes 1, 3, 4, 5, 6b, 7f, 9v, 14, 19f, and 23f.

Intraperitoneal versus intrapulmonary bacterial challenge. Mortality was assessed after intraperitoneal and intrapulmonary *S. pneumoniae* challenge (Table 2). Seven-day mortality rates were similar for both challenge routes among all 10 serotypes tested. Death invariably followed the detection of bacteremia within 24 h.

Rate of acquisition of bacteremia. The rate of acquisition of bacteremia was recorded after both intraperitoneal and intrapulmonary inoculation with *S. pneumoniae* serotype 6B. Mortality was assessed as well. Bacteremia with type 6b was acquired at the same rate regardless of challenge route, although mortality occurred 4 to 6 h later after intrapulmonary inoculation than after intraperitoneal inoculation (Table 3).

Intrapulmonary inoculation. Twelve pups were challenged with intrapulmonary injections of 0.5% agarose alone. No adverse effects were noted; on day 7 postinoculation, all pups were thriving. Histologic sections of lung demonstrated no pathology except occasional minimal hemorrhage at the site of the needle puncture.

Serotypes 1, 3, 4, 5, 6b, 7f, 9v, 14, 19f, and 23f were used as intrapulmonary challenge inocula. Histologic sections of lung from representative low- and high-numbered serotype (1, 6b, 14, 19f, 23f)-challenged animals demonstrated polymorphonuclear and leukocyte infiltration at the site of lung puncture and patchy infiltrative consolidation of lung parenchyma (Fig. 1). Lung homogenate cultures from representative low- and high-numbered serotypes demonstrated growth of corresponding *S. pneumoniae* types from 10^3 to 10^8 CFU/g of lung tissue following challenge inoculation of 1 to 10^3 organisms. Lung histology was normal and lung homogenate cultures were sterile in rats that received intraperitoneal inoculation with *S. pneumoniae*.

Sequelae of intrapulmonary inoculation with *S. pneumoniae*. Using rat passaged *S. pneumoniae*, intrapulmonary challenge with low inocula resulted in bacteremia, meningitis, and death (Table 4). Again, bacteremia was invariably followed by death of the animals. Table 1 shows data summarizing the inocula that produced 50 to 90% bacteremia for 10 serotypes. We found that the inoculum (CFU per rat) required to produce bacteremia in 90% of animals was 1 logarithm higher than that required to produce bacteremia in 50% of animals (data not shown).

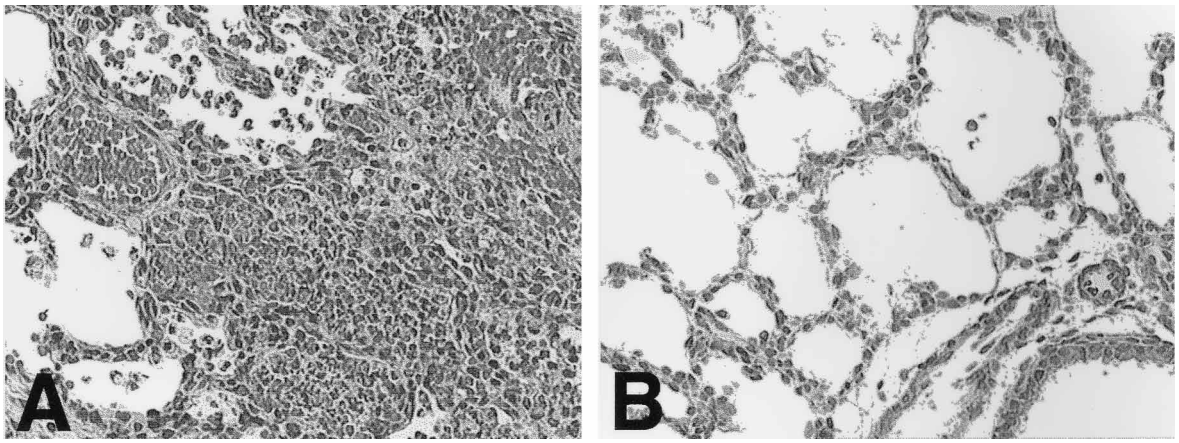

FIG. 1. Hematoxylin-eosin stain preparation of lung sections (magnification, ×200). (A) Congestion and infiltration of inflammatory cells, including neutrophils seen within a small bronchiole; (B) normal lung after intrapulmonary injection with 0.5% low-melting-point agarose.

TABLE 3. Acquisition of bacteremia after inoculation with 4 CFU of serotype 6b[a]

Route	No. of animals with bacteremia/no. alive (mortality rate)						
	4 h	6 h	12 h	20 h	24 h	30 h	36 h
Intraperitoneal	1/6	1/6	6/6	4/4 (2/6)	2/2 (4/6)	— (6/6)	
Intrapulmonary	0/6	1/6	5/6	5/6	4/5 (1/6)	1/2 (4/6)	0/1 (5/6)

[a] Acquisition of bacteremia was similar for both routes of infection; all bacteremic animals subsequently died. The mortality rate is indicated in parentheses (no. of deaths/total no. of animals).

DISCUSSION

We have developed a reliable model of low-inoculum *S. pneumoniae* intrapulmonary infection in infant rats. We were able to reduce the bacterial challenge, compared to initial high inocula used, necessary to produce bacteremia by animal passage of our strains. This effect has been noted in other models of bacterial infection (5, 9), but not to the magnitude that we achieved. Although we are unsure of the mechanism, the markedly increased virulence of the *S. pneumoniae* serotypes in our model after animal passage seems to be unique to passage in the infant rat.

Low-inoculum challenge doses of animal-passaged bacteria produced bacteremia and death after both intraperitoneal and intrapulmonary injection; virulence was similar regardless of route. In addition, the rates at which bacteremia was acquired after inoculation were similar for both routes, demonstrating that the intrapulmonary route was a reliable method of inoculation of low-challenge doses of *S. pneumoniae*. Uniformly, the inoculum that produced 90% bacteremia was 10-fold higher than that necessary to produce 50% bacteremia.

Although a reliable method of achieving bacteremia, intraperitoneal inoculation does not produce a focal infection site that is typical for *S. pneumoniae*. In contrast, intrapulmonary inoculation produced pulmonary inflammation and consolidation seen in histologic sections, and lung homogenate cultures grew the challenge serotypes of *S. pneumoniae*. Cultures of the lungs were obtained 24 h after *S. pneumoniae* challenge, when, based on our experience, most animals were bacteremic. We presume that bacteremia was a result of pneumonia, since high numbers of pneumococci (e.g., 10^3 to 10^8) were recovered from the lung, indicating replication of bacteria within the lung parenchyma and bronchioles. The intrapulmonary inoculation therefore produced not only pneumonia but also the sequelae of pneumococcal infection, i.e., bacteremia, meningitis, and death, more closely replicating disease in humans than the intraperitoneal inoculation.

TABLE 4. Intrapulmonary injection with low inocula results in bacteremia, meningitis, and death[a]

Serotype	Inoculum (CFU/rat)	Day 1		Day 7 mortality rate (no. dead/total)
		No. of bacteremic rats/total	No. of rats with meningitis/total	
1	1	3/4	3/4	3/4
	10	4/4	4/4	4/4
6b	1	4/12	1/12	4/12
	10	11/12	2/12	12/12
9v	20	8/9 (3 dead)	5/9	11/12
14	5	2/4	2/4	2/4
	50	3/3 (1 dead)	3/3	4/4
19f	5	2/4	0/4	2/4
	10	5/12	2/12	7/12
23f	0.2	4/8	2/8	4/8
	2	4/8	2/8	7/8

[a] Representative data from experiments using low- and high-numbered serotypes.

For all serotypes tested, we demonstrated that low-inoculum intrapulmonary challenges of *S. pneumoniae* produced reliable bacteremia, meningitis, and death. We feel that this model of *S. pneumoniae* infection in infant rats will be useful for the study of the pathophysiology of pneumococcal infections and for evaluating the protective capacity of immunization with antipneumococcal antibodies, including antibodies to the capsular polysaccharides and the common proteins such as pneumolysin, surface protein A, and the 37-kDa surface adhesin protein.

ACKNOWLEDGMENTS

This work was supported by Global Programme for Vaccines grant V23/181/92 from the World Health Organization (Geneva, Switzerland).

We are grateful to Michelle Ferretti for her comprehensive care of the animals and meticulous attention to detail before, during, and following these experiments. We also thank Amy Imrick for her expert instruction in dissection and preparation of lung specimens.

REFERENCES

1. **Alexander, J. E., R. Lock, C. A. M. Peeters, J. T. Poolman, P. R. Andrew, T. J. Mitchell, D. Hansman, and J. C. Paton.** 1994. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. Infect. Immun. **62:**5683–5688.
2. **Alonso DeVelasco, E., B. A. T. Dekker, A. F. M. Verheul, R. G. Feldman, J. Verhoef, and H. Snippe.** 1995. Anti-polysaccharide immunoglobulin isotype levels and opsonic activity of antisera: relationship with protection against *Streptococcus pneumoniae* infection in mice. J. Infect. Dis. **172:**562–565.
3. **Austrian, R.** 1981. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. Rev. Infect. Dis. **3**(Suppl.)**:**S1–S17.
4. **Breiman, R. F., J. S. Spika, V. J. Navarro, P. M. Darden, and C. P. Darby.** 1990. Pneumococcal bacteremia in Charleston County, South Carolina. Arch. Intern. Med. **150:**1401–1405.
5. **Briles, D. E., M. Nahm, K. Schroer, J. Davie, P. Baker, J. Kearney, and R. Barletta.** 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. J. Exp. Med. **153:**694–705.
6. **Friedland, I. R., and G. H. McCracken, Jr.** 1994. Management of infections caused by antibiotic-resistant *Streptococcus pneumoniae*. N. Engl. J. Med. **331:**377–382.
7. **Hoffman, J., M. S. Cetron, M. M. Farley, W. S. Baughman, R. R. Facklam, J. A. Elliott, K. A. Deaver, and R. F. Breiman.** 1995. The prevalence of drug-resistant *Streptococcus pneumoniae* in Atlanta. N. Engl. J. Med. **333:** 481–486.
8. **Kayhty, H., H. Ahman, P. R. Ronnberg, R. Tillikainen, and J. Eskol.** 1995. Pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine is immunogenic in infants and children. J. Infect. Dis. **172:**1273–1278.
9. **MacLeod, C. M., and M. R. Krauss.** 1950. Relations of virulence of pneumococcal strains for mice to quantity of capsular polysaccharide formed in vitro. J. Exp. Med. **92:**1–9.
10. **McClinn, S., and D. Williams.** 1996. Incidence of antibiotic-resistant *Streptococcus pneumoniae* and beta-lactamase-positive *Haemophilus influenzae* in clinical isolates from patients with otitis media. Pediatr. Infect. Dis. J. **15:** S3–S9.
11. **McCracken, G. H., Jr.** 1995. Emergence of resistant *Streptococcus pneumoniae*: a problem in pediatrics. Pediatr. Infect. Dis. J. **14:**424–428.
12. **Nielsen, S. V., U. B. S. Sorensen, and J. Henrichsen.** 1993. Antibodies against pneumococcal C-polysaccharide are not protective. Microb. Pathog. **14:**299–305.
13. **Pomat, W. S., D. Lehmann, R. C. Sanders, D. J. Lewis, J. Wilson, S. Rogers, T. Dyke, and M. P. Alpers.** 1994. Immunoglobulin G antibody responses to polyvalent pneumococcal vaccine in children in the highlands of Papa New Guinea. Infect. Immun. **62:**1848–1853.
14. **Ramisse, F., P. Binder, M. Szatanik, and J. M. Alsonso.** 1996. Passive and active immunotherapy for experimental pneumococcal pneumonia by polyvalent human immunoglobulin or $F(ab')_2$ fragments administered intranasally. J. Infect. Dis. **173:**1123–1128.
15. **Shapiro, E. D., and R. Austrian.** 1996. Serotypes responsible for invasive

Streptococcus pneumoniae infections among children in Connecticut. J. Infect. Dis. **169:**212–214.
16. **Siber, G. R.** 1994. Pneumococcal disease: prospects for a new generation of vaccines. Science **265:**1385–1387.
17. **Tart, R. C., L. S. McDaniel, B. A. Ralph, and D. E. Briles.** 1996. Truncated *Streptococcus pneumoniae* PspA molecules elicit cross-protective immunity against pneumococcal challenge in mice. J. Infect. Dis. **173:**380–386.
18. **Vella, P. P., S. Marburg, J. M. Staub, P. J. Kniskern, W. Miller, A. Hagopian, C. Ip, R. L. Tolman, C. M. Rusk, and L. S. Chupack.** 1992. Immunogenicity of conjugate vaccines consisting of pneumococcal capsular polysaccharide types 6b, 14, 19f, and 23f and a meningococcal outer membrane protein complex. Infect. Immun. **60:**4977–4983.
19. **Voss, L., D. Lennon, K. Okesene-Gafa, S. Smeratunga, and D. Martin.** 1994. Invasive pneumococcal disease in a pediatric population, Auckland, New Zealand. Pediatr. Infect. Dis. J. **12:**873–878.
20. **Zangwill, K. M., C. M. Vadheim, A. M. Vannier, L. S. Hemenway, D. P. Greenberg, and J. I. Ward.** 1996. Epidemiology of invasive pneumococcal disease in southern California: implications for the design and conduct of a pneumococcal conjugate vaccine efficacy trial. J. Infect. Dis. **174:**752–759.

Editor: V. A. Fischetti