Systemic Group B Streptococcal Disease in the Neonate: Characterization of an Oral Colonization Model Using the Suckling Rat

Leonard E. Weisman,*1 LuAnne McKinney,2 and Raphael Villalobos1

1Departments of Pediatrics, Uniformed Services University, Bethesda, MD 20814-4799, U.S.A., and Walter Reed Army Medical Center, Washington, D.C., U.S.A., and 2Department of Animal Pathology, Walter Reed Army Institute of Research, Washington, D.C., U.S.A.

(Accepted for publication, June 7, 1990)

Abstract Aspiration or ingestion of contaminated amniotic fluid or vaginal secretions has been suggested as a cause of systemic group B streptococcal (GBS) infection in the neonate. Suckling rat studies disagree on whether systemic disease will develop after an oral challenge of GBS. Our goal was to determine if systemic GBS disease would occur following oral colonization in the suckling rat and the effect of bacterial, host and environmental factors. Suckling rat littermates received oral inoculation on one of the first four days of life with varying doses and strains of GBS. Studies confirmed gastric inoculation without aspiration. Mortality and bacteremia decreased with age, increased with dose, varied with strain, and increased with asphyxia. Autopsy confirmed sepsis, intestinal colonization, meningitis, and pneumonia. Bacteremia was associated with an abnormal immature:total neutrophil ratio at 24 hr, thrombocytopenia at 48 hr, and neutropenia at 72 hr after inoculation. GBS can cause systemic infection in the host after oral colonization which appears age-, dose, strain-, and environment-dependent. Evaluation of GBS entry in the susceptible host may facilitate therapies directed toward preventing mucosal invasion.

Group B streptococcus (GBS) infection continues to produce serious neonatal morbidity and mortality. It has been suggested that early-onset (1, 3) and late-onset (15) GBS disease may be acquired following ingestion or aspiration of GBS-infected amniotic fluid or vaginal secretions. Though the suckling rat has been used to study GBS pathogenesis, prevention, and therapy, in these studies GBS was inoculated either intraperitoneally (11, 19), intranasally (8, 11), or subcutaneously (12, 25). Previous investigators using a suckling rat model disagree on whether systemic disease will develop following oral challenge with GBS (11, 16). The objective of this study was to determine if systemic GBS disease would occur fol-

Abbreviations: CFU, colony-forming unit; GBS, group B streptococcus; I/T, immature to total neutrophil ratio.
lowing oral colonization in the suckling rat and to evaluate the effect of bacterial, host and environmental factors on the development of this disease.

MATERIALS AND METHODS

Organisms. SS878 (virulent, type III clinical isolate strain), D136C (non-virulent, type III laboratory standard strain), 090 (type Ia laboratory strain), and 090R (a laboratory mutant strain devoid of Ia capsule and developed from strain 090) were kindly provided by Dr. Gerald W. Fischer, at the Uniformed Services University, Bethesda, Md., U.S.A. GBS types Ia and III were selected for these studies because together they are responsible for 45% of early-onset and 92% of late-onset infections (3). Before each experiment, organisms were incubated on a fresh blood agar plate for 18 hr, grown to mid-log phase in Todd-Hewitt broth (THB) as determined by optical density, then sedimented by centrifugation and resuspended to the desired concentration with normal saline. The actual number of colony-forming units (CFU) used was confirmed by serial dilution and quantitative plating (24).

Animals. Pathogen-free 14 day timed-pregnant outbred primigravida Wistar rats were obtained from a commercial breeder (Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.), housed separately and given antibiotic-free food and water ad libitum. All dams were sera negative for GBS type Ia and III antibody using both opsonophagocytosis (12) and enzyme-linked immunosorbent (19) assays. Dams without detectable antibody activity were selected for study, because type-specific antibody (12) decreases susceptibility to GBS infection and transplacentally acquired antibody might effect pup survival. At birth each litter was randomly divided into one of 4 orally inoculated groups, a subcutaneously inoculated group, and a control group. Only litters with 6 to 15 pups were used. Throughout the study, pups remained with their dams and were allowed to suckle ad libitum.

Oral inoculation. Pups were given 15 μl of concentrated GBS, receiving either one dose of 10⁷, two doses of 10⁷, one dose of 10⁸, or two doses of 10⁸ CFU. These concentrations of GBS were selected because they represented those found in amniotic fluid of women with chorioamnionitis (9). The volume was selected because it was proportional to the amount of amniotic fluid aspirated from a human neonate's stomach at delivery (20). Pups were allowed to suck on the pipette tip and drink the inoculum to avoid aspiration or traumatizing their mucosa. Animals receiving two doses were fed 2 hr apart. To validate gastrointestinal inoculation, 60 littermate pups were fed a dose of GBS as stated above or via a 2 french soft silastic catheter that was passed through the mouth into the stomach. Placement of the catheter in the stomach was confirmed visually. The catheter was flushed with air to insure complete delivery of inoculum to the stomach. Mortality of both groups was identical. Light microscopic and visual inspection of the oral cavity, esophagus, and stomach of several animals in both groups confirmed no evidence of tissue injury by either method. Further experiments showed no escape of methylene blue administered by gavage before or after asphyxia.
Subcutaneous inoculation. Pups were injected with 25 µl of concentrated GBS (10^4 CFU) just cephalad to the tail (12).

Age at inoculation. Litters were randomly selected for inoculation on one of the first four days of life. Pups weighed (mean±standard deviation) 6.0±0.6, 6.8±0.7, 7.8±0.8, 8.8±1.0 g, respectively on the first to fourth days of life just prior to inoculation.

Asphyxia and cold stress. Immediately after oral inoculation, randomly selected litters of 1-day-old pups were placed in a container of dry ice (CO2) for 5 min. This time was selected because it was required for the animal to become totally cyanotic and limp. Pups had totally recovered color and activity within 15 to 20 min.

Blood cultures. Tail bleeds of 10 µl were obtained from all pups at 6, 24, 48, 72, 96, and 120 hr after inoculation. Blood was cultured in 1.0 ml of THB at 37°C for 24 hr and then plated on to Columbia blood agar plates and incubated at 37°C for up to 72 hr. Bacteria were identified on the basis of typical colony morphology, staining characteristics, and beta-hemolysis on blood agar. Selected cultures were identified by lack of inhibition by bacitracin, and the CAMP test. Selected cultures of beta-hemolytic streptococcus were grouped serologically (10).

Leukocyte, platelet, and differential counts. Blood was collected via cardiac puncture and pups euthanized at 6, 24, 36, 48, and 72 hr after oral inoculation with two doses of 10^8 CFU of SS878 at birth in randomly selected litters and placed in EDTA-coated tubes. Leukocyte and platelet counts were done using a hemocytometer. Differential counts were done by counting 200 cells on peripheral blood smears using a Wright's stain according to the morphology of Chervenick et al (7). Blood cultures were also done on these samples. A comparison of hematologic parameters was performed between three groups: 1) noninfected pups, 2) infected but blood culture negative pups, 3) infected and blood culture positive pups, at each of the time intervals (6, 24, 48, and 72 hr after infection) evaluated.

Histologic examination. Several litters were randomly selected for histologic examination at 24 and 48 hr after oral inoculation with two doses of 10^8 CFU of SS878 at birth. Noninfected healthy and subcutaneously inoculated littermate controls were also examined. All animals had quantitative cultures obtained from blood via cardiac puncture, from gastric and small intestinal aspirate, from lung, liver, and brain (organ specimens were homogenized with 1 ml of THB) (24). The animals were then fixed in formalin and specimens microscopically examined after staining with hematoxylin-eosin, and Brown-Brenn. The specimens were prepared and interpreted by a single observer (LM) blinded to the pup's group.

Statistical analysis. The Fischer exact test was used to make comparisons of proportions. The independent t-test with Bonferronnii correction was used to compare means.

The study was approved by our institute Animal-Use Committee.
RESULTS

Effect of Postnatal Age

The postnatal-age-dependent mortality and bacteremia of 184 suckling rats, one to four days old, inoculated orally with 2 doses of $10^8$ CFU of strain SS878 is summarized in Fig. 1. Bacteremia and mortality increased with decreasing age. Increased mortality and bacteremia with decreasing postnatal age was also seen with smaller doses of GBS (not shown here). Of 50 controls (non-inoculated littermates), the mortality was 4% and none of these had GBS bacteremia. All subcutaneously inoculated pups ($n=44$) died with GBS bacteremia within 48 hr. Animals orally infected with lethal doses of GBS displayed a nonspecific illness pattern of lethargy and anorexia by 24 hr, paleness and labored respirations by 48

![Fig. 1. The postnatal age (one to four days) of suckling rats fed two doses of $10^8$ CFU of group B streptococcal strain SS878 affects cumulative mortality and bacteremia (vs. day one: $\star<0.001$, $\dagger<0.01$).]
hr, and death usually by 72 hr and no later than 96 hr. Most pups inoculated on
day 1 had not yet suckled, but suckling prior to inoculation appeared to have no
effect on mortality or bacteremia in this group (data not shown).

Effect of Dose

The dose-dependent GBS mortality and bacteremia of 164 one-day-old suckling
rats orally inoculated with either 1 or 2 feeds of $10^7$ or $10^8$ CFU of strain SS878
is summarized in Fig. 2. Bacteremia and mortality increased with increasing dose.
More than 50% of bacteremic pups had positive cultures by 24 hr after oral
inoculation.
Effect of Bacterial Strain

The strain-dependent GBS mortality and bacteremia of 142 one-day-old suckling rats orally inoculated with 2 feeds of $10^8$ CFU of one of four GBS strains is summarized in Fig. 3. Bacteremia and mortality varied with strain used.

Effect of Asphyxia and Cold Stress

Asphyxia and cold stress significantly increased mortality (76% vs. 51%, $P<0.03$, $n=43$) and bacteremia (89% vs. 66%, $P<0.01$) in 1-day-old suckling rats after oral inoculation with two feeds of GBS strain SS878. Asphyxia and cold stress without oral inoculation in littermates had no effect on survival; only two of 40 pups (5%) died and none had GBS bacteremia.
Pathology

The postmortem examination of 20 pups is summarized in Table 1. The degree of inflammation and quantity of organisms cultured from each organ appears related to the mode of inoculation. Pups subcutaneously inoculated had a greater mortality, earlier and more rapidly progressive clinical symptoms, and more severe pathologic findings (Table 1) than those orally inoculated. Pups sacrificed 48 hr after oral inoculation had more extensive and severe findings than those sacrificed 24 hr earlier. GBS grew from the gastric aspirate cultures of all orally inoculated pups at both 24 and 48 hr. No evidence of an inflammatory response was noted in the gastric muscosa or small intestine. Light microscope estimates of the quantity of GBS using the Brown-Brenn stain rarely observed organisms when quantitative cultures contained \( \leq 10^2 \) CFU of GBS. The uninoculated controls appeared to

---

Table 1. Estimate of inflammation (I) and CFU of group B streptococci at postmortem examination

<table>
<thead>
<tr>
<th>Method of inoculation</th>
<th>( n )</th>
<th>Age (hr) at exam</th>
<th>Blood CFU</th>
<th>Intestine I/CFU</th>
<th>Liver I/CFU</th>
<th>Lung I/CFU</th>
<th>Meninges I/CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>48</td>
<td>0</td>
<td>0/( \leq 10^2 )</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Oral(^a)</td>
<td>5</td>
<td>24</td>
<td>( 10^4 )</td>
<td>0/( \leq 10^2 )</td>
<td>1/( 10^3 )</td>
<td>0/( \leq 10^2 )</td>
<td>0/( \leq 10^2 )</td>
</tr>
<tr>
<td>Oral(^b)</td>
<td>5</td>
<td>48</td>
<td>( 10^6 )</td>
<td>0/( \leq 10^2 )</td>
<td>3/( 10^5 )</td>
<td>1/( 10^2 )</td>
<td>1/( 10^2 )</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>5</td>
<td>24</td>
<td>( 10^7 )</td>
<td>ND(^b)</td>
<td>4/( 10^6 )</td>
<td>3/( 10^3 )</td>
<td>1/( 10^4 )</td>
</tr>
</tbody>
</table>

\(^a\) 2 doses of \( 10^8 \) CFU of SS878 on day 1.

\(^b\) ND, not done.

0-4=Visual estimate (0=none, 1=few, 4=many).

---

Table 2. Platelets and neutrophils are decreased in one-day-old suckling rats fed two doses of \( 10^8 \) CFU of GBS

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>Hours after infection</th>
<th>Platelets/ml ( \times 10^4 )</th>
<th>Neutrophils/ml ( \times 10^4 )</th>
<th>I/T Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>6</td>
<td>411 (49)</td>
<td>2.54 (21)</td>
<td>0.31 (.03)</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>6</td>
<td>386 (59)</td>
<td>2.57 (22)</td>
<td>0.29 (.03)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>6</td>
<td>351 (47)</td>
<td>2.62 (30)</td>
<td>0.35 (.04)</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>24</td>
<td>491 (46)</td>
<td>1.88 (63)</td>
<td>0.05 (.02)</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>24</td>
<td>450 (50)</td>
<td>2.21 (56)</td>
<td>0.20 (.03)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>24</td>
<td>275 (41)</td>
<td>1.46 (18)</td>
<td>0.63 (.04)*,( # )</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>36</td>
<td>591 (86)</td>
<td>4.85 (1.4)</td>
<td>0.12 (.02)</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>36</td>
<td>322 (40)</td>
<td>2.92 (.78)</td>
<td>0.30 (.04)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>36</td>
<td>265 (43)</td>
<td>1.20 (.18)</td>
<td>0.52 (.04)*,( # )</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>48</td>
<td>595 (56)</td>
<td>2.90 (.42)</td>
<td>0.08 (.02)</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>48</td>
<td>555 (53)</td>
<td>1.88 (.35)</td>
<td>0.20 (.03)</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>48</td>
<td>113 (30)*,( # )</td>
<td>1.00 (.23)</td>
<td>0.48 (.07)*,( # )</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>72</td>
<td>574 (83)</td>
<td>2.71 (.28)</td>
<td>0.08 (.02)</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>72</td>
<td>546 (78)</td>
<td>2.25 (.30)</td>
<td>0.16 (.03)</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>72</td>
<td>68 (19)*,( # )</td>
<td>0.35 (.08)*,( # )</td>
<td>0.24 (.07)</td>
</tr>
</tbody>
</table>

Group 1, No infected, blood culture negative; group 2, orally infected, blood culture negative; group 3, orally infected, blood culture positive.

\( P \leq 0.05 \) when (*) for group 3 vs. 1, and (\#) for group 3 vs. 2.
develop minimal intestinal colonization without systemic disease.

**Thrombocytopenia and Neutropenia**

Quantitative platelet and neutrophil counts, and immature to total neutrophil (I/T) ratios are summarized in Table 2. Orally infected but culture-negative pups tended to have hematologic parameters intermediate between noninfected and culture-positive littermates, though they were not statistically different from the noninfected pups. Bacteremia was associated with an abnormal I/T ratio by 24 hr, thrombocytopenia by 48 hr, and neutropenia at 72 hr.

**DISCUSSION**

Previous reports disagree on whether systemic disease will develop after an oral challenge of GBS in the suckling rat. Ferrieri et al (11) observed none while Kim et al (16) reported 31% of suckling rats developed systemic disease following oral inoculation with GBS. Our data demonstrates greater than 66% of suckling rats develop systemic GBS infection following oral colonization. Both previous studies used older pups (≥3 days old). Though we used Wistar and the others used Sprague-Dawley pups, the mortality of 3-day-old pups fed 2 doses of $10^7$ CFU in our study (11%) was similar to that in Kim’s study (9.6%). Though it is difficult to accurately compare these studies, since doses and strains of GBS as well as strains and ages of pups used were different, it is clear that systemic GBS disease will develop in the suckling rat following oral inoculation.

The age, dose, and GBS-strain-dependent related mortality and bacteremia we observed in suckling rats fed GBS is similar to subcutaneously inoculated suckling rats (25). In human neonates, earlier gestational age and increased colonization are associated with increased GBS infection (18), while the impact of GBS strain has been implied by the strain-variable production of neuraminidase (17), adhesion to vaginal (5) and neonatal buccal epithelial cells (6), and susceptibility to opsonization by antibody (21). Though the effect of age, dose, or strain may be secondary to an immune system that is not fully developed (23), it may be further complicated by a specific lack of mucosal immunity (14).

This study demonstrates, for the first time, that the environment of the host (asphyxia/cold stress) plays an important role in GBS disease. Asphyxia has been used to develop a rat model for neonatal necrotizing enterocolitis using *E. coli* or *Klebsiella* (4). Clinically, it has been suggested that neonates who later develop sepsis appear to have an increased incidence of asphyxia (13, 18). Asphyxia and or cold stress may enhance the crossing of organisms from lumen to the systemic circulation because of a loss of local immunity (14).

The differences in platelet count, neutrophil count, and I/T ratio between infected nonbacteremic pups and control pups were unexpected. Though the differences were not statistically significant, the infected nonbacteremic pups consistently developed hematologic values between the bacteremic and control pups. We can only speculate about several potential mechanisms for these hematologic
changes: 1) bowel wall inflammation due to local organism invasion without septicemia or local toxin effect as seen in patients with necrotizing enterocolitis (4); 2) systemic absorption of GBS toxin as seen in animal models of GBS-induced pulmonary hypertension (3); or 3) our blood cultures did not detect low levels (≤10 CFU/ml) of bacteremia (though this is unlikely) (23). Many clinical reports describe the associated hematologic abnormalities of thrombocytopenia, neutropenia, and increased I/T ratios in patients with septicemia (1, 3). Whether human neonates, colonized but not bacteremic with GBS, also have mild hematologic abnormalities as observed in our suckling rats requires further study. This observation may provide, however, some explanation for the numerous hematologic abnormalities observed in neonates with suspected GBS sepsis without bacteremia.

The entry of GBS into the host, in this model, takes place through the gastrointestinal tract. Other organisms gain entry to the human via the gastrointestinal tract (4, 22), and Peyer’s patches is the site of penetration for such pathogens as reovirus and E. coli (2). While no one knows how GBS gains entry to the human neonate, using methods based in part on our experience, neonatal rhesus monkeys developed GBS septicemia following gastric inoculation (15). In light of all this, it seems likely that the gastrointestinal tract is a major site of GBS entry into the human neonate.

There appear to be multiple factors involved in the development of GBS disease including density of maternal colonization, type and strain of GBS, prematurity, presence of GBS type-specific opsonic antibody, complement, fibronectin, and local mucosal immunity. To produce systemic infection, GBS must penetrate the mucocutaneous barrier. Penetration may be facilitated by physical disruption of the barrier, high density of the bacteria, or ability to adhere to the barrier cells. Further study of the mechanism of entry of GBS in this model may lead to therapies directed toward preventing mucosal invasion of the susceptible host with this organism.

We gratefully acknowledge the advice of Dr. Gerald W. Fischer, Department of Pediatrics, Uniformed Services University, Bethesda, MD.

This research was funded in part by the Department of Clinical Investigation, Walter Reed Army Medical Center, Washington, D.C.

The opinions and assertions contained herein are those of the author and do not necessarily reflect those of the Department of the Army or the Department of Defense.


REFERENCES


(Received for publication, January 25, 1990)