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Chicken Embryo Model for Type III Group B Beta-Hemolytic Streptococcal Septicemia

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A lethal septicemia was induced in 11- and 12-day-old chicken embryos with intravenous inoculation of relatively small numbers of a clinical isolate (GBBHS-III-Bell) or a reference strain (GBBHS-III-D136-C) of group B beta-hemolytic streptococci (GBBHS). GBBHS-III-Bell was more virulent than GBBHS-III-D136-C, and 11-day-old chicken embryos were more susceptible than 12-day-old chicken embryos. Type-specific rabbit antisera protected the embryos from bacterial challenge, and this protective effect was absorbed with homologous but not heterologous GBBHS strains. A heterologous antiserum and normal rabbit sera provided some protection, which could be absorbed with either homologous or heterologous GBBHS strains. The chicken embryo is a suitable animal model for the study of infection and immunity with GBBHS type III.

Group B beta-hemolytic streptococci (GBBHS) are currently a major cause of serious perinatal infections (3). Five serotypes of GBBHS (Ia, Ib, Ic, II, and III) have been identified based on polysaccharide capsular and protein determinants (19). Although all of the serotypes are associated with infection, type III strains account for the majority of cases of neonatal meningitis (3, 4). Preliminary results suggest that the offspring of mothers who lack type III GBBHS antibodies are at greater risk of acquiring these infections (5). In vitro and in vivo studies have contributed considerably to the understanding of factors affecting GBBHS infections in humans (1, 6, 16, 19, 20). However, in the case of GBBHS-III, progress has been hindered because of the lack of an adequate in vivo experimental model. Numerous mouse passages of type III GBBHS have not led to sufficient virulization in mice (3), and one neonatal rat model required too large an inoculum of type III GBBHS to be suitable for studies of human immunity (M. H. Crumrine, G. W. Fischer, and M. W. Balk, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., Abstr. no. 151, 1976).

The chicken embryo is an excellent animal model for the study of infection and immunity to microorganisms pathogenic for humans (9). We have used the intravenous inoculation of 11- or 12-day-old chicken embryos to study GBBHS-III infections and related host defense mechanisms.

MATERIALS AND METHODS

Preparation of bacteria. GBBHS-III-Bell is a type III GBBHS strain isolated from a 7-day-old infant with meningitis. Reference strains of GBBHS type Ia-SS615 and type III-SS620 were obtained from the Center for Disease Control (Atlanta, Ga.) through the courtesy of Hazel Wilkinson, and strain GBBHS-Ia-SS615/28 is the former passed 28 times in adult mice for virulization by the method of Lancefield (19). GBBHS type III-D136-C was kindly supplied by Rebecca Lancefield of Rockefeller University, New York, N.Y. This strain is identical to GBBHS-III-SS620.

Samples of overnight cultures of these bacteria on Trypticase soy-5% defibrinated sheep blood agar (SBA) plates were inoculated into 100 ml of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) with 0.5% dextrose and grown at 37°C to mid-log phase and an optical density of 0.30 at 540 nm. Aliquots of 1.5 ml were placed in screw-cap glass tubes, frozen in an acetone-Dry Ice bath, and stored at −70°C until used.

For the chicken embryo injections, the frozen samples were rapidly thawed in a 56°C water bath and immediately placed on ice. The bacterial suspensions were serially diluted with phosphate-buffered saline, pH 7.4. Viability and concentration of the microorganisms in colony-forming units (CFU) per milliliter were determined by spread plating on SBA plates.

Collection and preparation of rabbit sera. Type-specific rabbit anti-GBBHS hyperimmune serum was prepared by the method of Lancefield (19), using New Zealand white rabbits and Formalinized whole cell vaccines of GBBHS-Ia-SS615 and GBBHS-III-SS620. Sera were heat inactivated for 30 min at 56°C, sterilized by passage through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.) and stored at −70°C in aliquots of 0.5 ml.

Absorption of sera. Sera were absorbed by Lancefield's method (19) using overnight cultures of GBBHS-Ia-SS615/28 or GBBHS-III-Bell in Todd-Hewitt broth, heat killed at 56°C for 30 min, and

packed by centrifugation. Sera were mixed in a 10:1 ratio with packed bacteria, incubated at 37°C for 30 min, centrifuged at 4°C, and sterilized by passage through 0.45-μm Millipore filters. Absorbed sera were stored at −70°C until used.

Inoculation of chicken embryos. Specific pathogen-free fertile eggs from White Leghorn chickens fed antibiotic-free meals (Spafas Inc., Roanoke, Ill.) were incubated in a Humidaire Incubator model N55, with automatic turning, controlled temperature (37.3 to 37.8°C), humidity (50 to 55%), and air circulation (Humidaire Incubator Inc., New Madison, Ohio). The age of the embryos was determined morphologically by using the Hamburger series of normal stages in the chicken embryo (15), with a minimum of three eggs per experiment sacrificed for this purpose. Eleven- and 12-day-old chicken embryos were selected for inoculation, the latter being easier to handle. Chicken embryos were inoculated intravenously following the method of Eichhorn (13) with minor modifications (7). Eggs were candled (B-B Candler; Val-A Co., Chicago, Ill.) to locate a straight, medium-sized chorioallantoic vein, and the direction of the blood flow was determined. A window (0.5 by 1.0 cm) was cut out of the shell using an electric hand drill (Mototool; Dremel Div., Emerson Electric Co., Racine, Wis.). The shell fragment was removed, and a drop of sterile light mineral oil was applied to the exposed shell membrane to render it transparent. Immediately before injection, a tuberculin syringe with a 27 gauge needle (1.25 in; ca. 31.8 mm) was filled with 0.1 ml of the bacterial suspension and an equal volume of phosphate-buffered saline or test serum, using an MLA automatic pipette (Medical Laboratory Automation, Inc., Mount Vernon, N.Y.). The syringe was briefly agitated, and 0.1 ml of the mixture was slowly injected in the direction of the blood flow under constant candling with a cool-light lamp (American Optical Corp., Buffalo, N.Y.). The needle was then slowly withdrawn to prevent hemorrhage, and the window was closed by replacement of the flamed shell fragment, covered with Parafilm (American Can Corp., Neenah, Wis.), and sealed with melted paraffin. The eggs were then placed horizontally with the window up in a Napco Incubator model J1640-10 (Scientific Products, McGaw Park, Ill.). Viability was assessed by candling at 5 h after the injection and then daily for the next 5 days. Embryos dying within 5 h of the injection were considered procedural pitfalls and were excluded from the experiment. Such early death occurred in only 1% of all eggs injected. In each experiment, the exact number of CFU injected into the embryos was established by spread plating 0.1 ml of two syringes containing bacteria with phosphate-buffered saline or test serum. Controls consisted of injections of the saline alone or a 1:1 dilution of the test serum in the saline. In the protection studies, 150 to 250 CFU of GBBHS-III-Bell were injected into 11- and 12-day-old chicken embryos to achieve an inoculum size that would be approximately two to three times the 90% lethal dose (LD_{90}; see below).

Distribution of GBBHS-III in the chicken embryo. Samples of blood, liver, and brain were obtained aseptically from three 12-day-old chicken embryos at 3, 6, and 10 h after inoculation with 100 to 200 CFU of GBBHS-III-Bell. The liver and brain samples were homogenized in tissue grinders. The CFU per 0.1 ml of blood or 100 mg of liver or brain were determined by spread plating on SBA plates. For histopathological studies, whole embryos were obtained at different time intervals after inoculation, fixed in Formalin, sectioned, and stained.

Statistical analysis of results. The 50% lethal dose (LD_{50}) and LD_{90} were determined by probit analysis, and the significance of the differences between results was calculated by the standardized normal deviate (2).

RESULTS

The susceptibility of 11- and 12-day-old chicken embryos to intravenous challenge with GBBHS-III-Bell or GBBHS-D136-C is shown in Table 1. GBBHS-III-Bell was extremely virulent, with 90% mortality observed when 11- and 12-day-old chicken embryos were inoculated with as few as 57 and 117 CFU, respectively. Eleven-day-old chicken embryos appeared to be more susceptible than 12-day-old embryos to GBBHS-III-Bell, the LD_{50} and LD_{90} calculated for 11-day-old embryos being significantly lower than those for 12-day-old embryos ($P < 0.01$ and $P < 0.05$, respectively) (Table 2).

GBBHS-III-Bell appeared more virulent than the reference type III GBBHS strain (GBBHS-III-D136-C) for 12-day-old chicken embryos with significantly different LD_{50} and LD_{90} values ($P < 0.001$). In the 11-day-old chicken embryos, only the LD_{90} ($P < 0.05$), but not the LD_{50}, was significantly different between the two GBBHS strains used. Irrespective of age, death occurred between 11 and 18 h after injection in chicken embryos inoculated with GBBHS-III-Bell and between 20 and 48 h when GBBHS-III-D136-C was used. The increased susceptibility to a bacterial challenge in 11-day-old chicken embryos as compared with 12-day-old chicken embryos was also observed with GBBHS-III-D136-C, although only the LD_{50} was significantly different ($P < 0.001$).

Peak bacterial multiplication occurred at 10 h, shortly before death (Fig. 1). Bacterial cultures in these series, as well as in samples from dead embryos, always consisted of pure colonies of the injected GBBHS strain. Microscopic examination of the liver, spleen, and lung revealed the predominance of bacteria in the vessels and reticuloendothelial system. In the mesonephros, large deposits of bacteria were found in the glomeruli and some in macrophages. Serial sections of the brain disclosed bacteria invading the meninges and the vascular endothelial cells of the brain tissue.

Rabbit anti-GBBHS III serum in a dilution as low as 1:100 protected 75% of 11-day-old and 94% of 12-day-old chicken embryos inoculated

TABLE 1. *Susceptibility of chicken embryos challenged intravenously with GBBHS-III-Bell and GBBHS-III-D136-C*

Strain	CFU injected	11-day-old embryos		12-day-old embryos	
		Dead/ injected	% Mortality	Dead/ injected	% Mortality
GBBHS-III-Bell	5–50	18/28	64	8/30	27
	51–100	33/34	97	8/14	57
	101–150	14/15	93	26/27	96
	151–200	6/6	100	55/56	98
	201–250	8/8	100	18/19	95
	>250	ND[a]	ND	32/32	100
GBBHS-III-D136-C	5–50	7/17	41	0/12	0
	51–100	7/8	88	2/8	25
	101–500	10/11	91	7/16	44
	501–1,000	10/11	91	7/8	88
	1,001–10,000	9/9	100	8/8	100

[a] ND, Not done.

TABLE 2. *Lethal doses*[a]

Embryo	GBBHS-III-Bell		GBBHS-III-D136-C	
	LD_{50} (CFU)	LD_{90} (CFU)	LD_{50} (CFU)	LD_{90} (CFU)
11-day-old	(1) 11 (5–24)[b]	(3) 57 (33–99)	(5) 25 (10–65)	(7) 269 (82–883)
12-day-old	(2) 39 (28–55)	(4) 117 (88–156)	(6) 218 (119–400)	(8) 1,198 (393–3,653)

[a] See Table 1. Statistical analysis of results is as follows: (1) versus (2), $P < 0.01$; (3) versus (4), $P < 0.05$; (5) versus (6), $P < 0.001$; (7) versus (8), not significant; (1) versus (5), not significant; (3) versus (7), $P < 0.05$; (2) versus (6), $P < 0.001$; (4) versus (8), $P < 0.001$.

[b] Paired numbers in parentheses indicate 95% limit of confidence.

with 150 to 250 CFU of GBBHS-III-Bell (Table 3). Protection declined to 43% in 11-day-old chicken embryos and 62.5% in 12-day-old chicken embryos, using a 1:1,000 dilution of the serum. There was no protection using a 1:10,000 dilution of the serum. Although rabbit anti-GBBHS-Ia serum or normal rabbit serum provided some protection (≤50%) when used in a 1:10 dilution in both 11- and 12-day-old chicken embryos, the protective effect disappeared when using a 1:100 dilution of the serum in 11-day-old embryos and a 1:1,000 dilution in 12-day-old embryos. Five out of ten normal rabbit sera diluted 1:10 showed some protection. However, no serum protected more than 50% of the embryos. A representative of these sera is shown in Tables 3 and 4.

Passive immunity provided by rabbit anti-GBBHS-III serum was absorbed with homologous but not heterologous bacteria (Table 4). In contrast, the protective effect observed with rabbit anti-GBBHS-Ia serum or normal rabbit serum was absorbed by either GBBHS-III or GBBHS-Ia bacteria.

DISCUSSION

All five serotypes of GBBHS have been associated with human disease, although GBBHS-III is responsible for over 90% of the cases of late-onset neonatal infections and early-onset neonatal meningitis (3, 4). This striking predominance of GBBHS-III in perinatal infections is in contrast to the equal distribution of the five serotypes in early-onset sepsis as well as in colonized women and neonates (4). Unlike GBBHS-I and II strains (3, 19, 20), GBBHS-III is strikingly nonvirulent in mice (3). In our own laboratory, 50 passages of GBBHS-III-SS620 in mice did not result in selection of bacteria significantly virulent for mice. GBBHS-III was also found to be of low virulence in neonatal rats (Crumrine et al., Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., Abstr. no. 151, 1976), although recently sepsis and meningitis have been induced in infant rats using as few as 11 CFU of a clinically isolated GBBHS-III strain (P. Ferrieri, personal communication). Still, the absence of a suitable animal model has hindered the progress of our knowledge of host defense mechanisms involved in GBBHS-III infections.

The chicken embryo provides an excellent model for the study of the virulence of microorganisms pathogenic for humans, such as *Neisseria gonorrhoeae* and *N. meningitidis* (10, 12, 14). Depending upon the route of inoculation,

the lesions frequently resemble those found in the natural host (9). We have found the chicken embryo to be an equally useful and reproducible model for the study of GBBHS-III infection and immunity. Eleven and 12-day-old chicken embryos were susceptible to the intravenous inoculation of relatively small numbers of both a clinical isolate and a reference strain of GBBHS-III, leading to lethal septicemia. GBBHS-III-Bell was more virulent than GBBHS-III-D136-C, and 11-day-old chicken embryos were more susceptible than 12-day-old chicken embryos.

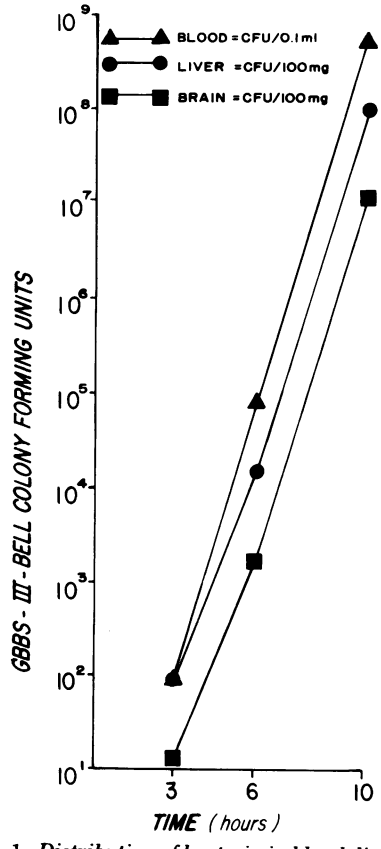

FIG. 1. *Distribution of bacteria in blood, liver, and brain of 12-day-old chicken embryos inoculated with 100 to 200 CFU of GBBHS-III-Bell.*

Type-specific rabbit anti-GBBHS-III serum protected chicken embryos from a lethal GBBHS-III-Bell challenge, and this protective effect was absorbed with homologous but not with heterologous bacteria. Anti-GBBHS-Ia and normal rabbit sera protected a smaller number of embryos, and this effect was absorbed with either GBBHS-III or GBBHS-Ia organisms. Baltimore et al. (6) found that unabsorbed rabbit antiserum to type Ia opsonized type III strains in a phagocytic assay. These results suggest that anti-GBBHS-Ia and normal rabbit sera contain

TABLE 3. *Protection by rabbit sera in chicken embryos injected with GBBHS-III-Bell*

Serum dilution injected	11-day-old embryos		12-day-old embryos	
	Survived/ injected	% Protection	Survived/ injected	% Protection
Anti-III				
1:10	14/14	100	27/28	96
1:100	12/16	75	16/17	94
1:1,000	12/28	43	10/16	63
1:10,000	0/7	0	0/8	0
Anti-Ia				
1:10	4/16	25	8/16	50
1:100	0/8	0	1/18	6
1:1,000	ND[a]	ND	0/8	0
NRS[b]				
1:10	3/16	19	10/28	37
1:100	0/12	0	5/20	25
1:1,000	ND	ND	0/4	0

[a] ND, Not done.
[b] NRS, Normal rabbit serum.

TABLE 4. *Effect of absorption of rabbit sera on the protective activity in chicken embryos injected with GBBHS-III-Bell*

Serum injected[a]	Absorbing strain	11-day-old embryos		12-day-old embryos	
		Survived/ injected	% Protection	Survived/ injected	% Protection
Anti-III	None	14/14	100	27/28	96
	GBBHS-Ia-615/28	8/8	100	11/12	92
	GBBHS-III-Bell	1/13	8	0/8	0
Anti-Ia	None	4/16	25	8/16	50
	GBBHS-Ia-615/28	0/6	0	1/14	7
	GBBHS-III-Bell	0/9	0	0/8	0
NRS[b]	None	3/16	19	10/28	36
	GBBHS-Ia-615/28	0/9	0	0/8	0
	GBBHS-III-Bell	0/9	0	0/8	0

[a] 1:10 dilution.
[b] NRS, Normal rabbit serum.

protective factors reactive with an antigenic determinant common to GBBHS-III and GBBHS-Ia strains. This common antigen could be the group B carbohydrate antigen or an unidentified antigen shared by the two strains. The protective activity of normal rabbit sera probably results from natural exposure to such antigenic structures.

Although 11- and 12-day-old chicken embryos have passively acquired maternal 7S immunoglobulins, active antibody production has not begun, and the complement system has not yet developed at this age (8). On the other hand, the phagocytic system is present and functionally adequate at this age (7, 21). Passively administered opsonins are capable of enhancing phagocytosis of *N. meningitidis* (14, 22), *N. gonorrhoeae* (10, 12), *Salmonella*, and smooth strains of *Escherichia coli* (17). The protection provided by anti-GBBHS-III sera and to a lesser extent by anti-GBBHS-Ia and normal rabbit sera is presumably due to specific opsonins which allow the embryo to clear more efficiently an otherwise lethal challenge of bacteria.

The chicken embryo is the first suitable animal model developed for the study of infection and immunity to GBBHS-III in humans. This in vivo method has potential advantages over other currently available in vitro techniques for the study of virulence of GBBHS type III organisms and for elucidating their highly selective pathogenicity in humans.

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