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Mucin Model for Group B Type III Streptococcal Infection in Mice

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An experimental murine infection was established by the intraperitoneal injection of a log-phase culture of a laboratory reference strain of *Streptococcus agalactiae*, Lancefield group B, type III (strain SS620), suspended in sterile hog gastric mucin. The enhancement of streptococcal virulence was measured by a significantly increased mortality in outbred ICR Swiss mice. An inbred C57BL6 strain of mice was resistant to the mucin-bacterial combination. Mucin, treated with Desferal to chelate the iron, did not lose the capacity to enhance the virulence of group B, type III streptococci in ICR Swiss mice. Iron-dextran was not a suitable substitute for mucin and failed to enhance the virulence of group B, type III streptococci. The results of these studies indicate that iron is not the resistance-lowering factor in this group B, type III streptococci-mucin model.

Streptococcus agalactiae, Lancefield group B, type III, is an opportunist of increasing importance in human infections, especially as the etiological agent of neonatal meningitis, osteomyelitis, and sepsis (8, 9, 21). This same organism is avirulent for mice, an animal which has served as a model of infection for the other four types of group B streptococci (GBS): Ia, Ib, Ic, and II (11, 20). A mouse model of type III GBS (GBSIII) would be useful for studies of the host-parasite relationship in this disease, for testing antibiotic susceptibility, and for antibody protection tests.

Mucin has been used to enhance infections in mice since the early studies of Nungester et al. (16). A mucin model used to produce experimental meningococcal infections in mice by Miller (14) was adapted to titrate protective antibody levels in sera (17) and remains a standard procedure today (5). A standardized mucin model is presently used by the Food and Drug Administration to select and test strains of *Salmonella typhi* for vaccine potency (*Code of Federal Regulations for Biological Products*, Title 21, Sections 620.10-620.15, Revised, 1976). The murine model developed in this study involves the intraperitoneal injection of sterile hog gastric mucin mixed with a log-phase culture of the laboratory reference strain of GBSIII (SS620). The enhancement of streptococcal virulence is significant in ICR Swiss outbred mice but not in the inbred C57BL/6 line. Studies are in progress to determine whether mucin affects the bacteria or the host defense mechanisms; the latter is suggested by the fact that a strain variation in development of disease was observed in mice. Results of studies to determine whether the iron content of mucin is responsible for its potentiation of GBSIII virulence were contradictory to the results found in the meningococcal system (5). Mucin treated with Desferal to chelate the iron did not lose the capacity to enhance the virulence of GBSIII in ICR Swiss mice; however, the GBSIII-mucin-Desferal combination did increase the mortality of the C57BL/6 mice. Iron-dextran did not enhance the virulence of the GBSIII. A volume effect of the dose of injected bacteria was noted, and volumes from 0.25 to 1.0 ml were found to be statistically more effective in the demonstration of virulence by a given number of bacteria than was the 0.1-ml dose.

MATERIALS AND METHODS

Mice. Female mice (18 to 20 g) were obtained from Harlan Industries, Inc., Indianapolis, Ind. Preliminary studies were done using outbred ICR Swiss mice; definitive studies were carried out in the inbred C57BL/6 line.

Mucin. Pfaltz and Bauer (M32610) gastric mucin was prepared as modified from Wood and Smith (personal observations, 1959-61): 1 g of mucin was placed in a sterile mortar, covered with sterile pyrogen-free saline, and allowed to stand for 30 min. The material was rubbed with a pestle until free from lumps, and saline was added in small portions to 10 ml. The mucin was transferred to a 50-ml bottle and autoclaved for 15 min at 15 lb/in^2 (ca. 103 kPa). This preparation was refrigerated and mixed 1:1 with bacteria to give a final concentration of 5% mucin for intraperitoneal injection into mice.

Bacteria: *S. agalactiae* type III (strain SS620) and type Ia (strain SS615) were kindly supplied by H. W. Wilkinson (Center for Disease Control, Atlanta, Ga.). Each strain was passaged five times in mice prior to

testing for 50% lethal dose (LD_{50}) levels; the organisms were passaged once a month to assure the maintenance of virulence. For the mouse passage, 0.05 ml from a frozen culture (−70°C) in rabbit blood was added to 9.0 ml of Todd-Hewitt broth (Difco) supplemented with eightfold Na_2HPO_4 and dextrose according to the method of Baker and Kasper (2) (henceforth referred to as THBK). After 18 h of incubation at 37°C in 5% CO_2 and 95% air, 0.5 ml was transferred to fresh THBK and incubated for 4 h. Three mice were injected intraperitoneally with 0.5, 0.25, and 0.1 ml of the 4-h culture. The mouse that died within 24 h after injection of the lowest inoculum was autopsied; the thoracic cavity was opened aseptically, and heart blood was removed by aseptically cutting the apex of the heart. Three calibrated loopfuls (0.001 ml each) of heart blood were inoculated into 9 ml of THBK and grown for 6 to 8 h. This growth was used to inoculate several Trypticase soy blood agar slants (BBL Microbiology Systems, Cockeysville, Md.) and was then reincubated for Lancefield typing to confirm the recovery of the specific type of GBS. The Trypticase soy blood agar slants were incubated overnight, growth was washed from the surface with 1 ml of sterile defibrinated rabbit blood, a sample was streaked on a Columbia CNA agar plate (Difco) for purity check, and the culture was frozen (−70°C) as stock cultures.

For mouse mortality studies, the 0.05 ml of stock culture was inoculated into THBK and grown for 16 to 18 h. An inoculum of 0.5 ml was transferred to 9 ml of fresh THBK and incubated for 4 h. Serial 10-fold dilutions were used to assess colony-forming unit (CFU) counts on duplicate plates (Trypticase soy agar); mice were injected intraperitoneally with various doses of GBSIII depending upon the protocol for that experiment (0.1 to 1.0 ml). Mortality was recorded for 5 days.

Lancefield typing. GBS vaccine strains, protocol, and reference typing sera were kindly provided by R. R. Facklam and H. W. Wilkinson (Center for Disease Control). Typing sera were produced in rabbits by repeated injections of formalinized whole bacterial cell vaccines of GBS types Ia (SS615) and III (SS620) according to the method of Lancefield (10) as modified by Wilkinson (personal communication, 1978). Streptococcal typing was done according to the Lancefield precipitin test as modified into a ring precipitin test by Wilkinson and Eagon (22) for polysaccharide antigens. Antigen for the typing test was prepared from the GBS organisms recovered from mouse passages, grown in THBK overnight, and processed by the hot-HCl extraction method of Lancefield (10).

Iron study. Desferal (deferoxamine mesylate; C.I.B.A., Summit, N.J.) in 500-mg amounts was suspended in 2 ml of sterile saline and mixed with appropriate volumes of mucin, iron-dextran, or saline to give 40 mg of the Desferal chelating agent per ml according to the method of Calver et al. (5). Mixtures were shaken for 24 h before use. A control of Desferal and saline or Desferal and mucin without bacteria was used along with the corresponding test injection with bacteria in a total volume of 1 ml given intraperitoneally.

Iron-dextran (Imferon; Merrill-National, Cincinnati, Ohio), 250 μg/ml, was injected intraperitoneally into mice along with the challenge dose of the GBSIII, using the method of Calver et al. (5) to determine the potentiating effect of this experimental iron-containing compound on the normally avirulent organisms.

Statistical analysis. The LD_{50} was calculated from the equation for the line of best fit for the bacterial dose as related to mortality at 5 days. The bacterial dose was transformed into logarithms, and the Hewlett-Packard HP-97 program for linear regression was used. The Yates method of chi-square analysis was used to analyze data of dead and surviving animals for the different mouse strains, volumes of inocula, and bacterial suspending media: broth, mucin, mucin-Desferal, or iron-dextran. Probability levels are given, with $P < 0.05$ considered significant.

RESULTS

Bacterial strain variation in mouse virulence. After five mouse passages of each bacterium, the preliminary tests for bacterial virulence were done using ICR Swiss outbred mice. The results confirmed the reported virulence of type Ia (SS615) and the relative avirulence of type III (SS620) for mice (11, 20) (see Table 1).

Of the 241 animals that died during the period of this study, 219 (90.8%) died in the first 24 h, another 15 (6.2%) died before 48 h, 5 died (2%) before 72 h, and 2 (0.8%) died by 96 h. A 2-day mortality test, as used by Lancefield et al. (11) for the GBS, retrospectively would have included 97% of the observed mortality. A 5-day mortality test was selected for this study to assure the observation of all of the GBS-associated mortality. The ICR Swiss mice consistently died within the 2-day period, whereas a few deaths in the C57BL/6 mice were observed in days 3 and 4 after inoculation with GBSIII.

Mucin as a resistance-lowering factor. The combination of GBSIII and mucin lowered the dose of bacteria required to induce mortality in the ICR Swiss mice by a 4- or 5-log difference (Table 1). The LD_{50} for GBSIII-mucin in ICR Swiss mice is 9.33×10^1, which is significantly different from the LD_{50} of 1.5×10^6 for GBSIII in broth ($P < 0.001$).

Inbred mice were used for definitive studies to establish the mouse model in a genetically controlled host. C57BL/6 mice, known to have high levels of complement activity, were used (1). Female mice were chosen for ease in handling, although complement levels are routinely lower (4). The inbred C57BL/6 mice were as resistant to the GBSIII broth inoculum as the ICR Swiss mice were, requiring 10^6 CFU for significant mortality. The LD_{50} for C57BL/6 mice was 1.7×10^6 ($P > 0.98$). The inbred mice did not respond to the GBS-mucin combination with the increased mortality found in the ICR strain; in fact, the mortality was not significantly dif-

TABLE 1. *Mortality of ICR Swiss and C57BL/6 mice from GBS suspended in various media*[a]

Mouse strain	CFU	GBSIa-broth		GBSIII-broth		GBSIII-mucin		GBS-mucin-Desferal		GBS-iron-dextran	
		D/T	%M	D/T	%M	D/T	%M	D/T	%M	D/T	%M
ICR Swiss	10^7			14/21	68						
	10^6			15/27	55			5/5	100		
	10^5					3/3	100			4/13	30
	10^4					11/12	91			2/13	15
	10^3					16/20	80			0/5	0
	10^2	20/20	100			18/24	75	5/5	100		
	10^1	20/20	100			10/24	41				
	10^0	15/20	75			8/38	21				
Control	0	0/5	0	0/5	0	0/10	0	0/5	0	0/5	0
LD_{50}		<10		1.5×10^6		9.33×10^1		$<10^2$		3.47×10^5	
C57BL/6	10^6			53/89	58			14/15	93	12/18	66
	10^5			3/25	12	3/3	100			1/18	6
	10^4					2/5	40			0/12	0
	10^3					0/10	0	4/10	40		
	10^2					1/15	6	1/15	6		
	10^1					1/20	5				
	10^0					0/10	0				
Control	0			0/5	0	0/10	0	0/10	0	0/5	0
LD_{50}				1.74×10^6		3.09×10^7		7.24×10^4		6.46×10^6	

[a] Injected intraperitoneally in a 0.5-ml volume. D/T, Number of mice dead/total number of mice tested; %M, percentage of mortality at 5 days.

ferent from that seen in controls receiving GBSIII in broth. The LD_{50} for GBSIII-mucin in C57BL/6 mice was 3×10^7 ($P > 0.10$), indicating a resistant response by this mouse strain.

The host strain response to the GBSIII-mucin mixture was significantly different in the ICR Swiss than in the C57BL/6 mice ($P < 0.001$). The results of the GBSIII-mucin experiments are summarized in Table 1. The number of GBSIII CFU required for 40% mortality when combined with mucin and injected into C57BL/6 mice was 10^4 as opposed to the 10^1 inocula in the ICR Swiss mice. Lower concentrations of bacteria inoculated into the C57BL/6 mice gave variable results with very few deaths.

In two experiments, mice were bled from the retroorbital plexus at the time of bacterial injection, at 15-min intervals for the first hour, and again at 2 h after injection. Bacteria were found in the blood during the first 15 min and increased in number during the 2-h testing period. Mucin did not change the rate of entry of GBSIII into the blood stream; this observation is in agreement with previous findings for meningococci (13).

Paralysis was noted in several of the surviving inbred mice (1% of those receiving 10^4 to 10^6 bacteria), occurring 7 to 10 days after bacterial injection. Similar observations had not been made with the ICR Swiss mice, because most of these outbred mice were dead within 24 h after inoculation of GBSIII, and they were not routinely kept any longer than the 5-day test period.

Iron as a factor in lowering host resistance. To determine whether the enhancing effect of the mucin was due to its iron content, an attempt was made to remove the iron from the mucin by using Desferal, an iron chelating agent successfully used for this purpose by Calver et al. (5). The mucin-Desferal mixture in the ICR Swiss mice continued to produce 100% mortality when combined with as few as 10^2 CFU of GBSIII, the lowest bacterial concentration used (Table 1). In the inbred (C57BL/6) mice, this same mixture killed only 1 of 15 mice, similar to the mortality obtained with GBSIII-mucin ($P = 0.5$). The difference in mortality levels in the two strains of mice was not statistically significant when the mucin-Desferal was combined with 10^6 CFU of GBSIII. Although there appeared to be an increase in mortality in both strains of mice when the GBSIII was suspended in mucin-Desferal rather than in broth, this difference was only statistically significant in the C57BL/6 mice ($P = 0.02$), due to the small sample size in the group of ICR Swiss mice. Chelation of the iron in mucin by Desferal did not remove the enhancing effect of the mucin on GBSIII infection in the ICR Swiss mice. This chelating agent did not cause mortality when injected as mucin-saline-Desferal into 20 mice. The mucin-Desferal mixture appeared to enhance the virulence of the GBSIII in the C57BL/6 mice when compared to the mortality

seen in mice receiving the bacterial-broth suspension at 10^3 CFU; however, this difference in mortality was not statistically significant ($P > 0.10$). A significantly lower LD_{50} of 7.24×10^4 was calculated for this mucin-Desferal mixture in the inbred mice when compared with the LD_{50} of 1.74×10^6 for the bacteria in broth; this result indicates that a significant difference in mortality is to be expected.

Calver et al. (5) were able to substitute iron compounds as resistance-lowering factors in their experimental meningococcal system. Although iron-dextran appeared to be the most acceptable substitute for mucin in Calver's study, it did not enhance the GBSIII infection in either strain of mice in the present study (see Table 1). Mortality levels were comparable to those expected or obtained by injection of GBSIII-broth at similar bacterial CFU levels. Iron does not appear to be responsible for the enhancement of infection in ICR Swiss mice and does not enhance the GBSIII infection in C57BL/6 mice.

Volume effects. During the course of these experiments, the effect of variation in the volume of inoculum was noted. Mouse passages had been routinely done using 0.1-, 0.25-, and 0.5-ml inocula of undiluted or 10-fold dilutions of bacterial cultures; this protocol was carried over into the experimental design for the different doses of CFU to be used. In the iron studies, 1-ml inocula were used according to the method of Calver et al. (5). The larger volumes (0.5 to 1.0 ml) seemed more effective in killing mice with the same or fewer bacteria than smaller inocula (0.1 to 0.25 ml); this could be attributed to technical difficulty in injection of small volumes. A statistically significant volume effect was found for the C57BL/6 mice given 10^6 bacteria in 0.5-ml versus 0.1-ml volumes. More mice died when the inoculum was given in the larger volume ($P < 0.001$). No significant difference was found between the 0.5- and 0.25-ml doses. Statistically significant variation in mouse mortality was found between 1.0- and 0.5-ml volumes in the ICR Swiss ($P < 0.001$) and the C57BL/6 ($P < 0.03$) mice at 10^1 to 10^3 CFU of bacteria in the mucin study (see Table 2). In the mucin-Desferal experiments at 10^6 CFU of GBSIII, mortality was high and no statistical difference could be determined for the volumes of inocula. The LD_{50} calculated for the 1-ml dose was 1.9×10^3, and that for the 0.5-ml dose was 7.24×10^4, indicating that a variation in mortality is dependent upon the volume of the inoculum. The importance of using a constant volume of inoculum is demonstrated by these observations. All statistical analyses were done on the mortality results based upon a 0.5-ml total volume of bacteria suspended in broth, mucin, mucin-Desferal, or iron-dextran.

TABLE 2. *Effect of the volume of a GBSIII-mucin inoculum on mouse mortality*

Mouse strain	CFU	Mice dead/total with inoculum vol:	
		1 ml	0.5 ml
ICR Swiss[a]	10^3	7/8	1/4
	10^2	8/8	0/3
	10^1	4/8	0/3
C57BL/6[b]	10^3	4/5	0/5
	10^2	1/5	0/5

[a] $\chi^2 = 11.23$; $P < 0.001$.
[b] $\chi^2 = 4.27$; $P < 0.03$.

DISCUSSION

This study has confirmed the virulence of *S. agalactiae* type Ia (SS615) and the relative avirulence of the laboratory reference strain of type III (SS620) for mice as previously demonstrated by Lancefield et al. (11), Wennerstrom and Shutt (20), and Swearingen et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B81, p. 29). A mucin model for type III infection in mice was successfully developed in the outbred ICR Swiss line. An attempt to develop the mucin model in a genetically defined host (the C57BL/6 line) was unsuccessful; this inbred mouse strain did not prove to be susceptible to the resistance-lowering capability of the mucin when combined with the GBSIII. These findings are in agreement with those of previous investigators concerning strain variation in susceptibility to infection. Rake (17) and Webster (18) had to select susceptible strains of Rockefeller Institute mice, both Swiss and white-faced breeds, for use in experimental meningococcal infection.

Deaths of GBSIII-infected mice usually occurred within 24 h with very few (2.8%) occurring after 48 h. A 5-day mortality test was used; however, the only deaths to occur on days 3 and 4 after bacterial challenge were in the inbred strain of mice. Further studies are proposed to find an inbred strain of mice that is as susceptible to the mucin-GBSIII as the ICR Swiss outbred mice. Susceptibility to GBS infection might then be related to H-2 type or other known genetic trait.

Lancefield et al. (11) used an 18-h culture for mouse passages and recovered bacteria from the spleen, a procedure that required around 10^8 CFU for lethality even after 10 mouse passages when repeated by Wennerstrom and Shutt (20). By using a log-phase culture in the present study, we decreased the lethal dose of GBSIII

to 10^6 CFU, and an LD_{50} of $1.6 \pm 1 \times 10^6$ was observed. By using heart blood rather than spleen from the moribund mice as a source of bacteria recovered from mouse passages, we may have selected a more virulent organism. Further studies are proposed to recover the GBSIII from the first mice to die (estimated at 8 to 18 h), anticipating the selection of GBS with higher levels of virulence.

The role of mucin in enhancement of bacterial infection or in lowering host resistance mechanisms is the subject of disagreement among investigators. The Wilson Laboratories no longer make granular hog gastric mucin, and since mucins are not standardized, investigators must test each mucin to determine its effect in their systems. The Pfaltz and Bauer mucin was effective in enhancing the GBSIII infection in ICR Swiss outbred mice, as indicated by a significant increase in mortality at 10^0 to 10^2 CFU; the enhancement was not found in the inbred C57BL/6 mice. This observation suggests that, although the source of mucin may be important, one must test it in several host systems because the strain of host is also relevant.

Calver et al. (5) were able to replace mucin with iron in the establishment of meningococcal infection in mice; they also removed the enhancement effect of mucin by chelating the iron in the mucin with Desferal. Although the GBSIII seemed to behave like the meningococcal infection in enhancement with mucin (17), the use of iron-dextran (Imferon, 250 μg/ml) to replace the mucin did not enhance the GBSIII infection in the ICR Swiss or the C57BL/6 mice. The attempt to remove iron from the mucin using Desferal did not remove the enhancement of virulence of GBSIII. The GBS-mucin-Desferal combination enhanced the virulence of the GBSIII in the C57BL/6 mice in which the GBSIII-mucin combination had no effect. Desferal has been shown to chelate iron in tissues, setting up an iron deficiency in such hosts (15). An increased risk of infection is proportional to the severity of the iron deficiency; markedly iron-deficient hosts have impairment of phagocytic and immune responses, especially reduced bactericidal capacity (6), which directly correlates with deficiency of iron (19). The role of an iron deficiency in establishing infection in C57BL/6 mice requires further investigation. In a study in which rats were made iron deficient by diet control, the LD_{50} of a strain of *Streptococcus pneumoniae* injected intraperitoneally was 3-log units less than that in the control host (7). In the present study, a 3-log difference was found when the GBSIII-mucin was used as a control, and a 2-log difference was seen when the GBSIII-broth was used as a control, for the inbred mice with the GBSIII-mucin-Desferal combination.

As a result of these observations, the enhancement of virulence of the GBSIII in the ICR Swiss mice cannot be attributed to the iron content of the mucin. The lack of enhancement of GBSIII virulence in the inbred strain (C57BL/6) indicates that the meningococcal model is a different host-parasite relationship. Calver and colleagues used male HPB black mice derived from two C57 sources, so one could expect genetic traits similar to the C57BL/6 line used in the present study. Although genetic variation in the mice cannot be ruled out as the source of these variations in results between the meningococcal system and the present studies on the GBS, these findings could be the result of an inherent difference in the bacterial response to the mucin.

The volume effect of the bacterial inoculum noted in these studies has been pointed out in other mucin-related experiments. The original mucin techniques involved the use of 1- or 2-ml inocula in mice. McLeod (12) used 0.25, 0.5, and 1.0 ml of mucin-meningococci and found a volume effect reflected in minimal lethal doses of 8.3×10^4, 8.3×10^3, and 83, respectively, for those volumes. Baltimore et al. (3) required an inoculum of 1.0 to 1.2 ml to lower the LD_{50} of GBSIII to 10^4 in mice, using blood as the resistance-lowering factor. Volumes of 0.5 ml of GBSIII-mucin were very effective in enhancing virulence in the present study of GBS in ICR Swiss mice. A volume effect was found at the 0.1-ml level which could be due to technical difficulties, but the significant effect between the 0.5- and 1.0-ml levels found in both mouse strains with the lower concentration of bacteria is important in pointing out the need to keep the volume standard throughout the experiment.

The mucin-GBSIII model is proposed as a working model for infection of ICR Swiss mice with this normally avirulent organism. Further studies are planned to demonstrate the usefulness of this model in selecting vaccine strains and testing vaccine potency.

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