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Enhanced Susceptibility of Mice with Streptozotocin-Induced Diabetes to Type II Group B Streptococcal Infection

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Since diabetes mellitus predisposes adults to group B streptococcal (GBS) bacteremia, a murine model of streptozotocin-induced diabetes and type II GBS bacteremia was developed to assess certain immune factors which might influence susceptibility to infection. In diabetic mice, the 50% lethal dose for two strains of type II GBS was significantly lower (>1 $\log_{10}$ decrease in CFU per milliliter) than in control animals. This enhanced virulence of GBS for diabetic animals was associated with prolonged bacteremia, persistent sequestration of organisms in the splanchnic reticuloendothelial system, and a shift from splenic to hepatic clearance. Although immunization of control and diabetic animals resulted in high concentrations of type-specific serum antibody, it had no effect on late reticuloendothelial system sequestration in diabetics. In contrast, depletion of complement by treatment of mice with cobra venom factor blocked reticuloendothelial system clearance and resulted in fatal infection in both diabetic and control mice. These results indicate that neither type-specific antibody nor an intact complement system is adequate for effective clearance of type II GBS bacteremia in mice with experimentally induced diabetes. This clearance deficit could be the result of a defect in hepatocyte membrane receptors necessary for removal of this encapsulated microorganism.

Bacteremic group B streptococcal (GBS) infection in adults is rare in the absence of preexisting disease states known to affect immune competence. One such underlying disease is diabetes mellitus, and its association with GBS bacteremia first was descibed by Eickhoff et al. (11) in 1964. Subsequent reports have validated this association (3, 6, 8, 10, 15). In some series, up to 50% of adults with GBS bacteremia are diabetics. The reason for the apparent propensity of adult diabetics to develop serious GBS infections remains undefined.

Streptozotocin (SZ), an antibiotic isolated from *Streptomyces achromogenes* var. *streptozoticus*, has cytotoxic properties which produce irreversible damage to pancreatic β-cells (1). It will induce diabetes without ketoacidosis in rodents. To assess the possible influence of diabetes in enhancing susceptibility to GBS bacteremia, a murine model of SZ-induced diabetes was developed. Type II strains of GBS were selected for study because, in contrast to their relative minor role in neonatal infection, they are frequent isolates from adults with GBS bacteremia, especially those with associated meningitis (18). With this experimental model, the kinetics of reticuloendothelial system (RES) clearance of GBS bacteremia and the influence of type-specific antibody and complement in mediating this clearance was defined in normal and diabetic animals.

MATERIALS AND METHODS

Animals. Adult male mice (Charles River CD-1 strain) weighing 30 to 35 g were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The mice were given free access to a standard laboratory diet and water.

Induction of diabetes mellitus. SZ (U9889, lot 60, 273-5) was kindly supplied by W. E. Dulin of The Upjohn Co., Kalamazoo, Mich. SZ was dissolved in a citrate buffer (pH 4.5), and a dose of 200 mg/kg in 0.5 ml of buffer was injected intraperitoneally within 10 min of dissolution. Control animals received the same volume of buffer alone. For each experiment, animals were used 1 to 2 weeks after SZ injection.

Estimation of blood sugar. At 7 to 10 days after induction of diabetes mellitus by SZ injection, the control and SZ-injected animals were bled from the retroorbital plexus. Serum glucose was determined by the Glytel test, (Pfizer Inc., New York, N.Y.) an orthotoluidine thiourea acetic acid reagent. The glucose levels for control animals ranged from 90 to 155 mg/100 ml. Nonfasted SZ-injected mice with serum glucose levels greater than 300 mg/100 ml were considered diabetic.

Bacteria. Mouse-passed type II GBS strain, 18RS21, provided by the late Rebecca Lancefield, and a clinical

type II GBS isolate, strain 611, from an adult diabetic with bacteremia were employed. Strains were grown at 37°C to mid-log phase and stored in 0.5- to 1.0-ml portions at −20°C. For each experiment organisms were subcultured overnight in Todd-Hewitt broth, washed, and diluted in 0.9% NaCl to the desired number of CFU for inoculation. Quantitation of inoculum size was estimated by an optical density of 0.02 at 540 nm in a Coleman Junior spectrophotometer (model 6C; Coleman Systems, Irvine, Calif.) and was confirmed by enumerating serial dilutions of the inoculum on blood agar plates containing 5% sheep blood (Remel Regional Laboratories, Lenexa, Kans.).

Formalin-killed whole cell vaccines of the type II GBS strain 611 were prepared by the method of Lancefield et al. (14). Vaccine diluted in 0.9% NaCl at a dose of 6.3×10^{11} CFU/ml was administered intravenously (0.5 ml) in one group of animals and intraperitoneally (1.0 ml) in another at three successive weekly intervals.

Antibody determination. Concentrations of antibody to native type II GBS polysaccharide were determined by a radioactive antigen-binding assay. The antigen was purified type II polysaccharide free of immunological reactivity with the group B determinant and intrinsically labeled with ^3H. Details of the assay have been described previously (D. L. Kasper, C. J. Baker, B. Galdes, E. Katzenellenbogen, and H. J. Jennings, submitted for publication).

Mouse lethality tests. Strains of type II GBS were tested for virulence in mice by a method similar to that of Lancefield et al. (14). Strains grown overnight in Todd-Hewitt broth contained approximately 5.0×10^8 CFU/ml. After washing, serial 10-fold dilutions were prepared in 0.9% NaCl, and 1.0 ml was injected intraperitoneally in groups of four animals each at a given dilution. Dead mice were counted and removed from their cages at 24-h intervals. All 50% lethal dose (LD_{50}) values represent the mean of two experiments and were calculated by the method of Reed and Muench (17) after 10 days of observation. Preliminary experiments indicated that the LD_{50} of type II GBS for control mice was identical when inoculation with organisms grown to mid-log phase was compared with a stationary phase (e.g., overnight) inoculum. Therefore, an overnight inoculum was employed for subsequent experiments.

Clearance studies. For clearance of bacteremia, mice received a dose of type II GBS, strain 611, intraperitoneally, and serial blood samples were obtained from the retroorbital plexus at 2, 6, 24, 72, and 96 h after injection. After dilution in sterile 0.9% NaCl, samples were inoculated onto blood agar plates and CFU were determined after overnight incubation at 37°C. For RES clearance studies, mice were infected intraperitoneally with type II GBS, strain 611. Challenged mice were observed daily for 7 days, and the number of deaths was recorded. Groups of four mice from each experimental group were sacrificed at 2, 6, and 24 h and at 3 and 7 days after challenge. The liver and spleen were removed aseptically, weighed, and homogenized in cold physiological saline. After preparation of serial 10-fold dilutions, 10 μl of each dilution was inoculated onto blood agar plates, and the number of CFU was determined after overnight incubation at 37°C.

In vivo complement depletion. Cobra venom factor (CVF) (lot 54111), partially purified venom of *Naja haje*, was obtained from Cordis Laboratories, Miami, Fla. Diabetic and control mice were injected intraperitoneally with 2 U each at 0, 8 and 24 h and were injected with bacteria 1 h after the last injection of CVF. Titers of C3 measured by radial immunodiffusion (16) were a mean of 5% of normal (range, 2 to 10%) at the time of bacterial inoculation.

TABLE 1. LD_{50} values of normal and diabetic mice infected with various doses of type II GBS

Group	Type II GBS strain	LD_{50} (CFU)
Diabetic[a]	18RS21	4.6×10^5
Control[b]	18RS21	4.8×10^6
Diabetic	611	8.7×10^6
Control	611	9.8×10^7

[a] Mean serum glucose concentration, 590 mg/100 ml; range, 300 to 800 mg/100 ml.

[b] Mean serum glucose concentration, 118 mg/100 ml; range, 90 to 155 mg/100 ml.

RESULTS

Lethality studies. Lethality tests were performed in diabetic and normal control mice after intraperitoneal injection with various doses of type II GBS. The LD_{50} with both the Lancefield strain, 18RS21, and the clinical type II GBS isolate, 611, was significantly lower (>1.0 $\log_{10}$ CFU/ml) in diabetic than in control mice (Table 1). Mortality was observed at a lower inoculum employing the mouse-passed strain, 18RS21, than with the fresh clinical isolate. For control mice, all deaths occurred within 72 h after challenge. However, in each experiment performed, 10 to 20% of the deaths among diabetic animals occurred between days 4 and 10 of infection.

Bacteremia. In view of the unexpected occurrence of late deaths in the diabetic group of mice, the course of bacteremia was quantitated sequentially after an intraperitoneal injection of type II GBS, strain 611, in groups consisting of four normal or diabetic animals each. The two groups of animals sustained similar degrees of bacteremia for the first 72 h after inoculation (Table 2). However, by 96 h after injection, each of the controls had resolution of bacteremia, whereas sustained bacteremia was observed in the diabetic animals and was associated with fatal outcome in two animals.

Clearance and localization of GBS in normal and diabetic animals. The patterns of RES clearance after intraperitoneal challenge were studied using a quantitative colony counting technique. Although the splanchnic RES patterns were initially similar for the two groups of animals, diabetic mice consistently had higher numbers of organisms in the liver (Fig. 1). After correc-

TABLE 2. Course of bacteremia in normal and diabetic mice after an intraperitoneal injection of type II GBS, strain 611 (4×10^7 CFU/ml)

Time after injection (h)	Mean CFU/ml (range)		No. bacteremic/total	
	Normal	Diabetic	Normal	Diabetic
2	4×10^5 (8×10^4–8×10^5)	1×10^6 (1×10^5–3×10^6)	4/4	4/4
6	3×10^6 (6×10^5–7×10^6)	1×10^6 (4×10^4–2×10^6)	4/4	4/4
24	6×10^5 (4×10^4–1×10^6)	2×10^6 (2×10^5–2×10^6)	4/4	4/4
72	7×10^5	7×10^5 (4×10^4–1×10^6)	1/4	2/2[a]
96		6×10^5 (1×10^5–1×10^6)	0/4	2/2[a]

[a] Each of two surviving mice remained bacteremic.

tion for organ weight, the distribution of CFU recovered indicated a shift to hepatic clearance in diabetic animals compared with controls at the 24-h sampling time (58 versus 13% of CFU recovered) (Table 3). At 3 days postchallenge, organisms were no longer recovered from the control animals, but a portion of the diabetic mice continued to harbor organisms in both the spleen and liver. At 1 week after injection, replication of organisms had occurred in the RES in two of four diabetic animals, and the CFU recovered approached that of the initial inoculum.

Effect of active immunization on clearance and localization of group B streptococci. Before induction of diabetes, sera were obtained from the retroorbital plexus of mice to determine the concentration of type II-specific antibody. Of 25 mouse sera tested in the radioactive antigen-binding assay, four (16%) were found to have type-specific antibody in moderate concentration (mean, 5.4 μg/ml; range, 2.5 to 7.4 μg/ml), whereas the remainder had very low levels (<2.0 μg/ml) of antibody to the type II capsular antigen. Immunization of animals with Formalin-killed type II GBS caused a significant (twofold or greater) increase in type II-specific antibody in eight (89%) of nine animals immunized intravenously (mean, 35.4 μg/ml; range, 2.9 to 92 μg/ml) and in each of 14 animals immunized by the intraperitoneal route (mean, 100.2 μg/ml; range, 13.5 to 276 μg/ml). Because of the simplicity and immunogenicity of intraperitoneal vaccine, animals employed in subsequent experiments were immunized by this route.

Experimental diabetes was induced in a group of animals with immunization-induced high levels of type II-specific antibody in their sera, and RES clearance of type II GBS by immunized and nonimmunized diabetic and control mice was determined. At 2 h after challenge, hepatic clearance was greater in immunized diabetic and immunized control animals (36 and 47% of CFU per gram, respectively) as compared with control animals (17% of CFU per gram) (Table 3). RES clearance was initially somewhat more rapid for immunized diabetic and nondiabetic animals than for nonimmunized animals (Fig. 2). However, as observed for nonimmunized diabetic mice, a portion of animals (25%) failed to clear the RES of organisms by 72 h, and the same percentage continued to harbor GBS in the spleen and liver at the 1-week sampling time. No tendency for more rapid clearance was observed

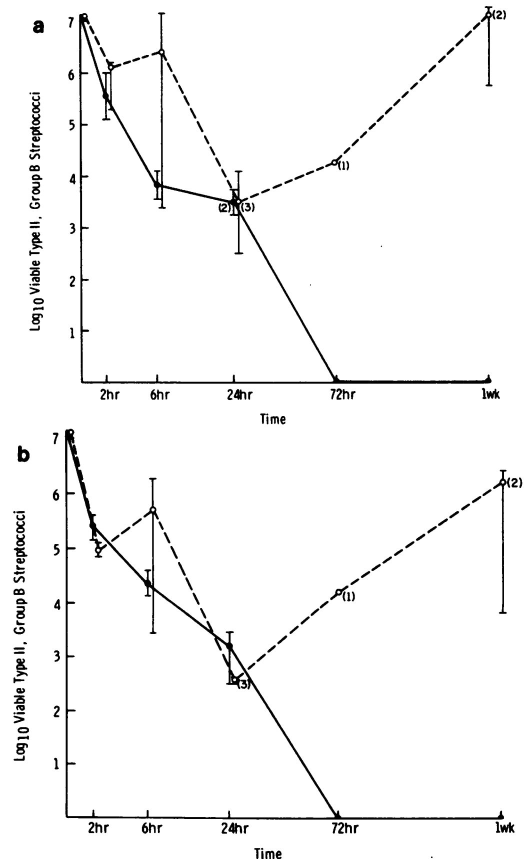

FIG. 1. Patterns of RES clearance of type II GBS in the liver (a) and the spleen (b) of normal (●) and diabetic (○) mice. Each point represents the mean and range of CFU in the whole organ for four mice or the number (within parentheses) from which type II GBS were persistent.

TABLE 3. Organ-specific clearance of type II GBS

Mice	Mean (range) % CFU recovered in liver [a]	
	2 h	24 h
Control	17 (12–22)	13 (3–29)
Diabetic	26 (18–45)	58 (35–81)
Immunized control	47 (16–58)	31 (8–54)
Immunized diabetic	36 (7–64)	38
CVF-treated control	30 (26–43)	55 (30–95)
CVF-treated diabetic	31 (19–49)	76 (55–99)

[a] CFU per gram of liver/CFU per gram of liver plus spleen.

for animals with extremely high serum antibody titers (>100 μg/ml) when compared with those with type II-specific serum antibody in moderate concentration (mean, 5 μg/ml).

Role of complement in clearance and localization of group B streptococci. Since the presence of a high concentration of type II-specific antibody before induction of diabetes had no effect on the persistence of bacteria localized to the splanchnic RES, the role of complement in modulating the clearance of type II GBS was then examined by comparing clearance patterns in control and diabetic animals to that of animals depleted of complement by administration of CVF. At 2 h after inoculation, RES clearance was similar for each group of animals (Fig. 3). At 6 h after injection of type II GBS, the clearance pattern of complement depleted control and diabetic mice paralleled that of diabetic mice. By 24 h, significant replication of type II GBS in the spleen and liver was observed for both CVF-depleted control and diabetic mice, and the majority of animals in these groups had died before the 72-h sampling time. As observed previously, splanchnic sequestration of type II GBS was undetectable in control mice at 72 h and 1 week after inoculation, whereas 25 to 50% of complement-sufficient, diabetic animals had continued replication of organisms (data not shown).

The organ-specific distribution of CFU recovered indicated a preponderance of hepatic clearance in both diabetic and control, complement-depleted animals at 24 h after inoculation (Table 3). After correction for organ weight, an average of 55 and 76% of CFU were recovered from the livers of CVF-treated control and CVF-treated diabetic animals, respectively.

DISCUSSION

Since the apparent enhanced susceptibility of adult diabetics to GBS bacteremia is understood poorly (7), the SZ-induced murine model of diabetes provided a system in which the effect of this disease state on host immune factors impor-

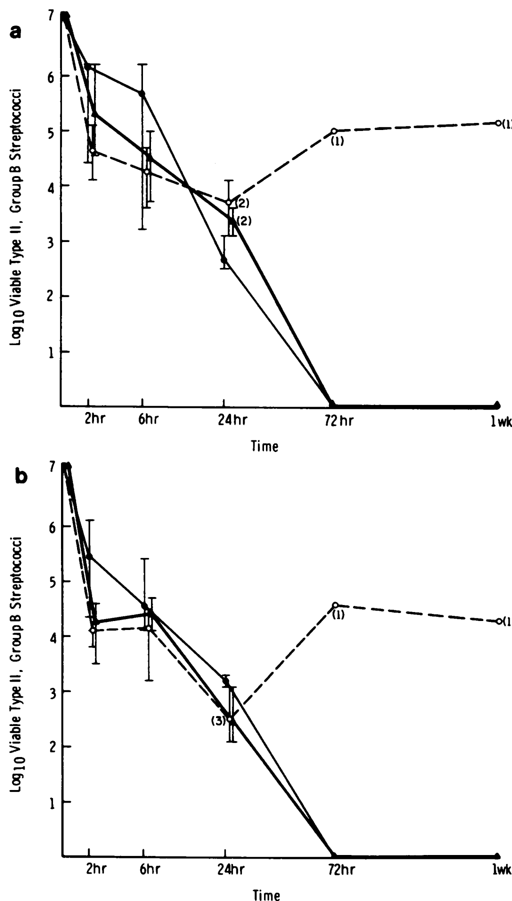

FIG. 2. Patterns of hepatic (a) and splenic (b) clearance of type II, GBS in immunized diabetic (○), immunized control (▲), and nonimmunized control (●) mice. Each point represents the mean and range of CFU in the whole organ for four mice.

tant in the pathogenesis of GBS infection could be examined. Initial experiments indicated type II GBS had increased virulence for diabetic animals. Although mortality was similar for 72 h after an LD_{50} challenge, diabetic, but not control, animals had deaths from days 4 to 10. These late deaths were associated with persistent bacteremia and prolonged sequestration of viable microorganisms in the hepatic and splenic RES. Similar findings have been noted in an experimental model of *Pseudomonas aeruginosa* infection in mice with SZ-induced diabetes (13). In that model, late deaths after inoculation were associated with increased numbers of organisms in liver, kidney, and, to a lesser extent, spleen in diabetic compared to control mice.

To determine the role of the splanchnic RES system in mediating clearance of type II GBS bacteremia from normal and diabetic mice,

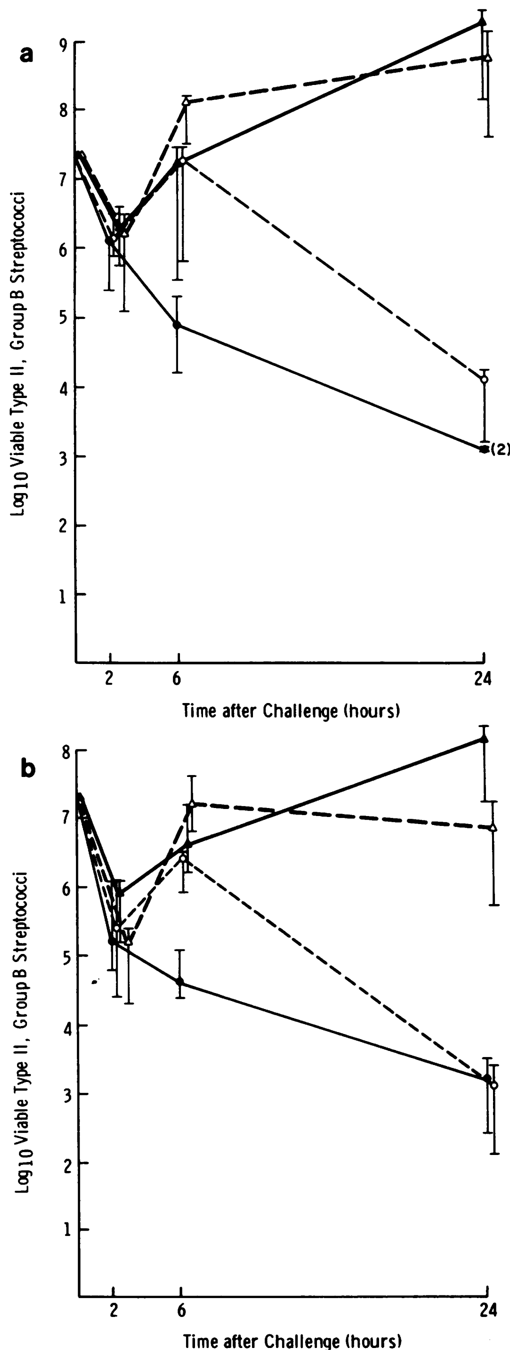

FIG. 3. Effect of complement depletion by CVF on patterns of hepatic (a) and splenic (b) clearance in normal and diabetic mice. Symbols: control mice (●), diabetic mice (○), CVF-treated mice (▲), and CVF-treated diabetic mice (△).

groups of animals were sacrificed at intervals after intraperitoneal inoculation, and viable organisms in the liver and the spleen were enumerated. Consistently higher numbers of organisms were recovered from the livers of diabetic animals, and a shift to hepatic clearance was observed in the diabetics at 24 h after inoculation. Furthermore, at 72 h and 1 week after LD_{50} challenge, 25 to 50% of diabetics, but not controls, had sequestration of viable GBS in both organs.

Immunization of mice with Formalin-killed type II GBS cells effected high concentrations of type-specific antibody and caused more efficient initial clearance of organisms in normal and diabetic animals. However, type II-specific antibody did not influence persistence of viable bacteria in the RES in a portion of diabetic animals at the 72-h and 1-week sampling times. This finding contrasts with that of Brown et al. (4) in a guinea pig model of experimental type 7 pneumococcal bacteremia. In that model, immunization of animals increased sequestration of pneumococci in the liver and was associated with resolution of bacteremia. However, in nonimmune animals, the relative increase in splenic clearance was also effective in clearing bacteremia. In the diabetic GBS model, hepatic clearance was somewhat greater in both diabetic and control immunized animals when compared with unimmunized controls. However, the increase in hepatic clearance did not effect resolution of bacteremia in immunized diabetic mice. This may relate to negation of the protection afforded by type-specific antibody by the diabetic state. This postulate is consistent with the findings of Kitahara et al. (13) in which the protective efficacy of immune serum against *P. aeruginosa* challenge was reduced in diabetic mice.

Since complement is important for clearance of bacteremia in the nonimmune host (12), its role in clearance of type II GBS bacteremia was determined by treating normal and diabetic animals with CVF. Initial RES clearance patterns of CVF-treated animals paralleled those of untreated diabetic mice. However, in both diabetic and control animals, depletion of complement produced a lethal defect of RES-mediated clearance. Compared with the findings in experimental pneumococcal bacteremia in which CVF treatment of guinea pigs was associated with greater sequestration of organisms in the spleen (5), no shift toward splenic clearance was observed in complement-depleted mice infected with type II GBS. At 24 h after challenge, hepatic clearance predominated in both CVF-treated and diabetic mice (Table 3). Therefore, an intact complement system is required for effective clearance of type II GBS bacteremia in both normal mice and those with SZ-induced diabetes.

The preceding experiments indicate that experimental diabetes in mice is associated with

increased susceptibility to type II GBS bacteremia. Infection in these animals is characterized by failure to clear bacteremia, sequestration of organisms in the splanchnic RES, and a shift from splenic to hepatic clearance. Although an intact complement system is essential for clearance of type II GBS from the bloodstream and type II-specific antibody enhanced initial clearance, neither was effective in preventing prolonged sequestration of viable GBS in the blood and splanchnic RES of diabetic animals. One hypothesis by which this defect in clearance might be explained is the decreasd binding capacity of hepatocytes for asialoglycoproteins which occurs in SZ-induced diabetes (2). Hepatic plasma membrane receptors normally bind and remove plasma proteins from the circulation after a desialation step which exposes galactosyl residues on the terminal end of carbohydrate chains (9). Since the native type II GBS capsular polysaccharide contains both sialic acid and galactose terminal residues, decreased binding of hepatic plasma membrane receptors for particles with these surface residues could contribute to the ineffective clearance. This mechanism would explain the persistent RES sequestration of type II GBS in experimentally induced diabetes.

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LITERATURE CITED

1. **Agarwal, M. K.** 1980. Streptozotocin: mechanisms of action. FEBS Lett. **120**:1–3.
2. **Ashwell, G., and A. G. Morell.** 1974. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. Adv. Enzymol. 41:99–128.
3. **Bayer, A. S., A. W. Chow, B. F. Anthony, and L. B. Guze.** 1976. Serious infections in adults due to group B streptococci. Clinical and serotypic characterizations. Am. J. Med. **61**:498–503.
4. **Brown, E. J., S. W. Hosea, and M. M. Frank.** 1981. The role of complement in the localization of pneumococci in the splanchnic reticuloendothelial system during experimental bacteremia. J. Immunol. **126**:2230–2235.
5. **Brown, E. J., S. W. Hosea, and M. M. Frank.** 1981. Reticuloendothelial clearance of radiolabelled pneumococci in experimental bacteremia: Correlation of changes in clearance rates, sequestration patterns, and opsonization requirements at different phases of the bacterial growth cycle. RES J. Reticuloendothel. Soc. **30**:23–31.
6. **Butter, M. N. W., and C. E. DeMoor.** 1967. *Streptococcus agalactiae* as a cause of meningitis in the newborn, and of bacteraemia in adults. Differentiation of human and animal varieties. Antonie van Leeuwenhoek J. Microbiol. Serol. **33**:439–450.
7. **Drachman, R. H., R. K. Root, and W. B. Wood, Jr.** 1966. Studies on the effect of experimental nonketotic diabetes mellitus on antibacterial defense. I. Demonstration of a defect in phagocytosis. J. Exp. Med. **124**:227–240.
8. **Duma, R. J., A. N. Weinberg, T. F. Medrek, and L. J. Kunz.** 1969. Streptococcal infections. A bacteriologic and clinical study of streptococcal bacteremia. Medicine **48**:87–127.
9. **Durand, G., J. P. Dumont, M. Appel, D. Durand, J. Davy, J. Féger, and J. Agneray.** 1980. Effect of streptozotocin diabetes on sialic acid content and glycoprotein binding of isolated hepatocytes. Horm. Metab. Res. **12**:247–251.
10. **Dworzack, D. L., G. R. Hodges, W. G. Barnes, and W. Rosett.** 1979. Group B streptococcal infections in adult males. Am. J. Med. Sci. **277**:67–73.
11. **Eickhoff, T. C., J. O. Klein, A. K. Daly, D. Ingall, and M. Finland.** 1964. Neonatal sepsis and other infections due to group B beta-hemolytic streptococci. N. Engl. J. Med. **271**:1221–1228.
12. **Fearon, D. T., and K. F. Austen.** 1980. The alternative pathway of complement—a system for host resistance to microbial infection. N. Engl. J. Med. **303**:259–263.
13. **Kitahara, Y., T. Ishibashi, Y. Harada, M. Takamoto, and K. Tanaka.** 1981. Reduced resistance to Pseudomonas septicaemia in diabetic mice. Clin. Exp. Immunol. **43**:590–598.
14. **Lancefield, R. C., M. McCarty, and W. N. Everly.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. J. Exp. Med. **142**:165–179.
15. **Lerner, P. I., K. V. Gopalakrishna, E. Wolinsky, M. C. McHenry, J. S. Tan, and M. Rosenthal.** 1977. Group B Streptococcus (*S. agalactiae*) bacteremia in adults. Analysis of 32 cases and review of the literature. Medicine **56**:457–473.
16. **Mancini, G., A. O. Carbonara, and J. F. Heremans.** 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry **2**:235–254.
17. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
18. **Wilkinson, H. W.** 1978. Analysis of group B streptococcal types associated with disease in human infants and adults. J. Clin. Microbiol. **7**:176–179.