Impaired Phagocytosis of Pneumococcus Type 3 in Splenectomized Rats[1] (36263)

W. D. BIGGAR,[2] D. BOGART, B. HOLMES,[3] AND R. A. GOOD[4]

Departments of Pediatrics, Microbiology and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

The spleen is known to be important for lymphopoiesis (1), phagocytosis (2), and the development of normal immunological defense mechanisms (3). That it has persisted in much the same general morphological state since its appearance in the primitive fishes (4), implies that it has a definite survival advantage. The contribution of the spleen towards the host reticuloendothelial (RE) system has been studied extensively. Despite this, the importance of the spleen in the host defense against bacterial invasion remains controversial. Since the observation of King and Shumacker (5) that splenectomized patients with congenital hemolytic anemia have an increased susceptibility to bacterial infection, several reports confirming their observation have appeared (6). The clinical picture is variable but may include fulminant bacterial sepsis, purulent meningitis, disseminated intravascular coagulation, and death within 24 hr. These patients appear to be at greatest risk 12 to 18 months after splenectomy (7, 8). Eraklis *et al.* (7), in a review of 467 splenectomized patients, described 3 groups with respect to their susceptibility to bacterial infection after splenectomy. The very low risk group included patients splenectomized for trauma or idiopathic thrombocytopenia. The minimal risk group included patients with hereditary spherocytosis and hypoplastic or aplastic anemia. The high risk group was comprised of patients with primary diseases involving their RE system (Gaucher's disease, portal hypertension, thalessemia, and Wiskott-Aldrich syndrome). It seems, from these observations, that the amount of RE system involvement prior to splenectomy will dictate, to some extent, the degree of risk from bacterial sepsis following splenectomy. The most frequent bacterial pathogens isolated from these patients appear to be the pneumococci, *Haemophilus influenzae,* and group A streptococci (6).

In contrast to these observations, others insist that no increase in susceptibility to infection follows splenectomy (8–15).

Animal models designed to elucidate this apparent conflict have encountered similar differences of opinion. Splenectomized rabbits (16), rats (17), and humans (18) have an impaired antibody response following the intravenous administration of heterologous erythrocytes. Leung *et al.* (19) clearly demonstrated that splenectomized rats have a greater mortality than do control rats following an intravenous challenge of Pneumococcus type 25. Furthermore, the spleen was found to be important for the initiation of the immune response to intravenously administered pneumococcal vaccine. Pneumococcal immunization, given intravenously 6 days prior to splenectomy, afforded good protection to the splenectomized rats compared to nonimmunized splenectomized rats. However, no protection was observed if the intravenous immunization was initiated in the absence of the spleen. Neonatally splenectomized mice showed increased susceptibility to

[1] Aided by grants from the National Science Foundation, American Heart Association. The National Foundation-March of Dimes and U.S. Public Health Service (AI-00798 and AI-08677).

[2] Queen Elizabeth II Canadian Research Fellow, Department of Pediatrics.

[3] Established Investigator of the American Heart Association.

[4] American Legion Memorial Research Professor, Regents' Professor of Pediatrics, Microbiology and Pathology.

early death under laboratory conditions that permitted exposure to much infection (20). Such splenic influence could not be demonstrated when mice, splenectomized early in life, were kept under ideal conditions of husbandry, which minimizes exposure to environmental pathogens (21, 22).

Many earlier investigators, however, have been unable to demonstrate a similar increased susceptibility when splenectomized animals were challenged by the various routes and with different bacteria (23–27).

The following investigation was undertaken to further evaluate this critical clinical problem. To this end, the *in vitro* phagocytosis of Pneumococcus type 3 by rat leukocytes in presence of serum from splenectomized rats was examined.

Materials and Methods. Charles River rats obtained from Midcontinent Research Animals Inc., Shawnee, KS, were used throughout. They were housed at 72°F and allowed free access to Purina rat chow and water. At 3 months of age the rats were splenectomized or sham operated. Splenectomy was achieved through a small lateral flank incision. The abdominal wall and skin were closed separately with 2-0 silk and skin clips, respectively. For the sham operation, a ligature was placed through the greater omentum using a similar surgical approach. No deaths were recorded with this procedure.

Preparation of Pneumococcus type 3. A Pneumococcus type 3 was maintained at 4° on Todd-Hewitt slants. Every 3–4 weeks the organism was passaged through mice to ensure virulence and encapsulation. New slants were inoculated with pneumococcus after the purity and type were determined. Pneumococci were prepared by an overnight incubation in Todd-Hewitt broth in a water-bath shaker at 37°. The bacteria were collected by centrifugation for 10 min at 1700*g* and resuspended in the appropriate dilutions to provide 5–10 $\times$ 10^6 organisms/ml in the stationary growth phase.

Serum samples. Blood was obtained from rats 5 days and 3 months after splenectomy or sham operation and allowed to clot. The sera obtained were used immediately, and then stored in small aliquots at —70° for future testing.

Polymorphonuclear leukocyte phagocytosis. Phagocytic studies of rat leukocytes were determined by the Maaløe method (28) with the modifications described by Cohn and Morse (29) and Hirsch and Strauss (30).

Rat peripheral leukocytes were prepared by heavy molecular weight dextran-sedimentation (Dextran Type 500, Sigma Chemical Co., St. Louis, MO) of heparinized blood. Blood (10 ml) containing 1 mg of heparin was mixed with 3 ml of 6% dextran in saline and incubated at room temperature for 30 min. The plasma containing leukocytes, platelets, and few erythrocytes was withdrawn. The leukocytes were centrifuged at 200*g* and washed with heparinized saline. The leukocytes were centrifuged, washed again, and resuspended in Hanks' balanced salt solution (HBSS, Microbiological Ass., Bethesda, Md) to give a concentration of 10 $\times$ 10^6 cells/ml.

Phagocytic tests were done in 13 $\times$ 75-mm Falcon plastic tubes (Falcon Plastics, Division of Bio-Quest Oxnard). Each tube contained 0.5 ml of leukocyte suspension, 0.1 ml of bacterial suspension, 0.1 ml of serum, and 0.3 ml of HBSS. Each assay included the serum from splenectomized rats and control leukocytes and control serum and control leukocytes. The tubes for assay of phagocytosis were incubated at 37° on a Lab Tech Tilter. Aliquots (0.1 ml) were removed at 0, 10, 30, 60, and 90 min. The time zero sample represents the actual number of bacteria added. Viable bacteria were quantitated by a standard dilution plate technique. In this assay, phagocytosis was completed by 60 min. The additional samples at 10, 30, and 90 min were done to observe the kinetics of phagocytosis and confirm any abnormality observed at 60 min. A sample is tested at 90 min to ensure that the bacteria are not multiplying when the assay is terminated.

Pneumococcal immunization. Overnight cultures of pneumococci (growth in Todd-Hewitt media enriched with sheep blood) were killed by adding formalin to a concentration of 0.5%. These were maintained at room temperature for 72 hr. The suspension was centrifuged at 1700*g* and resuspended in

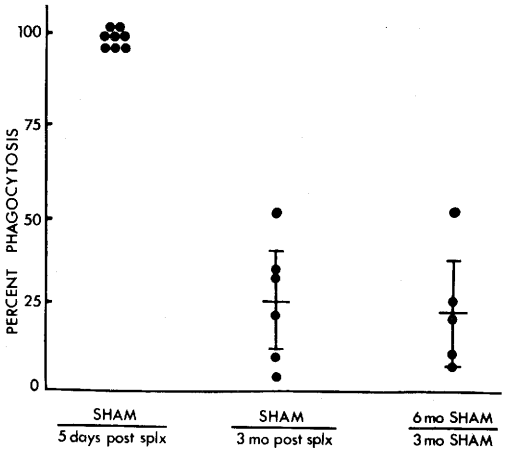

FIG. 1. Percentage phagocytosis of splenectomized rats: Percentage phagocytosis of pneumococcus by leukocytes using serum from rats splenectomized 5 days or 3 months previously; the percentage phagocytosis was calculated as the number of viable extracellular bacteria remaining in the control assay at 60 min divided by the number of viable extracellular bacteria remaining in the test assay (×100). Sham/3 months post splx.; mean, 23.2 ± SE, 7.1; 6 months sham/3 months sham; mean, 22.4 ± SE 7.6.

the appropriate dilutions to contain 10^8 bacteria/ml. This suspension (0.1 ml) was injected intravenously or subcutaneously. The sera were harvested 14 days after immunization and stored in aliquots at —70° for future testing.

Results. As illustrated in Fig. 1, serum from rats which were tested 3 months after splenectomy showed a marked deficiency of phagocytosis of type 3 Pneumococcus when compared to serum from age-matched sham-operated control rats [mean 23.2 ± 7.1 (SE)]. A deficiency was observed when the sera from splenectomized rats were tested with leukocytes from control rats, but no phagocytic defect was observed when normal rat sera were tested with leukocytes from splenectomized rats. When tested on different days, phagocytosis using the sera from control rats was similar (percentage phagocytosis: mean 99.6 ± 0.59). Heat-inactivated serum failed to support phagocytosis.

In contrast to the rats tested 3 months after splenectomy, serum samples from rats examined 5 days after splenectomy had a normal capacity to promote phagocytosis of pneumococcus. Serum from normal 6-month-old animals was much more effective than serum from normal 3-month-old rats whether tested with leukocytes from a 3- or 6-month-old rat.

TABLE I. *In Vitro* Phagocytosis of Pneumococci Using Immune and Nonimmune Rat Sera and Normal Rat Leukocytes.

Serum source	Phagocytosis[a] (%)
Sham/splx	50
Sham/splx iv_1	50
Sham/splx sc_1	118
Splx/splx iv_1	100
Splx/splx sc_1	200
Sham iv_1/splx iv_1	35
Sham sc_1/splx sc_1	82

[a] Percentage phagocytosis is calculated as the ratio of number of bacteria remaining in control serum divided by the number of bacteria remaining in test serum multiplied by 100. Ratios were calculated from 6 groups of 5 animals: sham; splx; sham sc_1; sham iv_1; splx sc_1; and splx iv_1. Abbreviations: splx = splenectomized; iv_1 = intravenously immunized; and sc_1 = subcutaneously immunized.

Table I compares the effects of specific intravenous and subcutaneous immunization on the capaicty to phagocytize pneumococcus *in vitro*. Splenectomized rats, injected intravenously, showed no improvement of their phagocytic defect compared to nonimmune sham-operated rats or splenectomized nonimmunized rats. Furthermore, this deficiency became more pronounced when these same rats were compared to the sham rats which had been immunized intravenously.

In contrast to the above observations, splenectomized rats which had been immunized subcutaneously, showed improved capacity to phagocytize pneumococcus. Most significant was the marked improvement when splenectomized rats immunized subcutaneously were compared to the nonimmunized splenectomized rats.

Discussion. To date, epidemiological analysis of splenectomized children and animal experiments designed to examine the effects of splenectomy on the host's defense mecha-

nisms, although suggesting vulnerability to pneumococcus and other organisms, have been inconclusive. These discrepancies may, in part, be due to the different organisms studied, to the various routes of administering the bacterial challenge and to comparing children with different diseases splenectomized at different ages.

The spleen is known to be important for the initiation of an antibody response to the intravenous administration of particulate antigen (17–18, 31) and for efficient clearing of certain bacteria from the circulation in the absence of opsonins (2).

Ellis and Smith (6) have reviewed the evidence that the spleen has a primary role in clearing intravenously administered particulate antigen in the absence of specific antibody. In contrast to the spleen, the liver is relatively inefficient in clearing particulate antigens in the absence of specific opsonins. When opsonic factors are available, the liver, and not the spleen, is of primary importance in the host's ability to clear that antigen from the circulation.

Our studies demonstrate that sera from young rats, tested 5 days after splenectomy show normal *in vitro* phagocytosis of pneumococci when compared to young sham-operated rats. This 5-day interval between splenectomy and testing would not appear to be of sufficient duration for the preexisting pneumococcal opsonins to be catabolized. Furthermore, without specific immunization, the sham-operated rats would not produce significantly greater quantities of pneumococcal opsonins over the 5-day interval. It is, therefore, not unexpected that sera of young splenectomized and young control rats are equal in their ability to support phagocytosis of pneumococci *in vitro*. That the serum opsonins for pneumococci increase significantly with age was demonstrated by comparing normal 3- and 6-month-old rats. Serum from the older rats had a much greater capacity to enhance *in vitro* phagocytosis of pneumococci compared to the younger rats. In these young animals, where pneumococcal opsonins are relatively deficient, the primary defense against an intravenous challenge of pneumococcus would reside in the spleen. In support of this was the finding that young rats, challenged intravenously 5 days after splenectomy, are deficient in their ability to clear pneumococci from the circulation (32). Furthermore, Leung *et al.* (19) have clearly demonstrated that young rats have a greatly increased susceptibility to an intravenous challenge of Pneumococcus type 25 administered soon after splenectomy.

Sutliff and Finland (33) found that children, during the first 2 years of life have deficient antipneumococcal immunity as compared to older children and adults. It is during this critical period of immunological development, when antipneumococcal antibodies are relatively deficient, that removal of the spleen would leave the young patient most vulnerable to pneumococcal sepsis. It would appear that the vulnerability of the young splenectomized host to pneumococcal bacteremia would be attributable not only to a serum opsonic deficiency incurred as a result of splenectomy, but, in addition, would be attributable to the inability of the liver to compensate for the loss of the spleen in the absence of significant antipneumococcal antibody. In support of this was the finding that, when pneumococci were opsonized prior to intravenous injection, the splenectomized rats cleared the pneumococci normally (32). Under these experimental conditions the liver could effectively clear the preopsonized bacteria in the absence of the spleen. The spleen therefore, in the absence of pneumococcal opsonins, is the primary host defense mechanism against that organism. In contrast to the rats tested 5 days after splenectomy, sera from rats examined 3 months after splenectomy were found deficient in supporting phagocytosis *in vitro*. That this deficiency was attributable to a defect of serum and not of neutrophil function was demonstrated. Serum from normal rats supported phagocytosis by neutrophils from splenectomized rats normally. This serum deficiency was clearly attributable to an impairment of the splenectomized rats and not to enhanced phagocytosis by control rats, since the number of bacteria phagocytozed using control sera was similar (mean 995,800 $\pm$ 650). Further definition of this serum deficiency will re-

quire studies of both specific, and nonspecific immunoglobulins in the deficient sera. The role of nonantibody serum factors could be defined with studies using agammaglobulinemic rat serum. Whether other bacteria in addition to Pneumococcus type III are affected by the deficiency is a critical question. Techniques to study *Haemophilus influenzae* are being developed since the use of chocolate agar pour plates has not given accurate viable bacterial counts.

During the second 3 months of life, it appears that the normal rat spontaneously develops serum factors necessary for enhancing the *in vitro* phagocytosis of pneumococcus. This was evidenced by the finding that serum from 6-month-old rats was far superior in opsonizing pneumococci than was serum from 3-month-old rats. The immunologic activation may be the result of exposure to pneumococci in the environment or possibly from immunization by cross reacting polysaccharide antigens (34, 35). The spleen then would appear to be important for the development of this "natural" immunity to pneumococci.

Experiments presented here, designed to compare the effects of intravenous and subcutaneous immunization of splenectomized rats, support the evidence that the spleen is crucial in initiating an antibody response to intravenously administered particulate antigens (17, 18, 31). Intravenous immunization of splenectomized hosts had no apparent beneficial effect on the capacity of serum to promote *in vitro* phagocytosis. This finding is in agreement with the work of Leung *et al.* (19), who were clearly able to demonstrate that intravenous immunization of splenectomized rats with killed pneumococci offered no increased protection for a subsequent intravenous challenge of viable organisms. However, splenectomized rats, immunized subcutaneously, produced serum factors capable of enhancing the *in vitro* phagocytosis of pneumococci. Indeed, serum from splenectomized rats, immunized subcutaneously, phagocytized pneumococci as effectively as did nonimmunized control rats.

There now exists sufficient experimental evidence to indicate that the material presented here is relevant to the clinical problem of splenectomy in children. Baker *et al.* (36) have shown IgM to be the primary immunoglobulin formed in response to pneumococcal antigen. Michael and Rosen (37) have demonstrated that the bactericidal and opsonizing properties of macroglobulins (IgM) are superior to those of IgG. Young children have low levels of circulatory IgM and are deficient in pneumococcal antibodies (18). Furthermore, splenectomized children have even lower levels of serum IgM compared to normal children. These factors would support the clinical observations that young children, with low levels of serum IgM and deficiencies of specific antipneumococcal antibodies are most susceptible to overwhelming sepsis by this organism in the first 18 months after splenectomy. It is during this period, in the absence of a functional spleen and inadequate serum opsonins to support the liver in its compensation, that the spleen seems most important. If the individual can survive this critical period, then he may develop specific opsonins perhaps as a result of oral and respiratory tract immunization, to provide the necessary serum opsonins for efficient phagocytosis by the liver. Preliminary evidence in splenectomized patients supports this hypothesis (38). If splenectomy is to be undertaken in children, particularly children under 2 years of age who have a high risk of sepsis, specific immunization of these patients with pneumococci and *Haemophilus influenzae* might reduce their overwhelming susceptibility to fatal infection by these organisms. Efforts to provide effective vaccines against organisms of multiple specificities seem warranted.

Summary. Splenectomy is frequently undertaken in patients as an adjunct to the medical treatment of clinical problems or subsequent to traumatic rupture. Several reports of an increased incidence of pneumococcal sepsis in these patients have been made. In contrast, others insist that no significant increase in susceptibility to infection follows splenectomy. Using an *in vitro* assay of pneumococcal phagocytosis by leukocytes, rats were found to have a serum deficiency of phagocytosis, 3 months after splenectomy.

Specific subcutaneous immunization of these splenectomized rats diminished the defect. No such improvement was seen, however, following intravenous immunization. It is suggested that subcutaneous immunization to pneumococcus or other antigens may offer a safe method of reducing the risk of bacterial sepsis in splenectomized patients.

We thank Sue Buron and Jim Wicks for their excellent technical assistance.

1. Auerbach, R., *in* "The Thymus in Immunobiology" (R. A. Good and A. E. Gabrielsen, eds.), p. 105. Harper & Row (Hoeber), New York (1964).

2. Saba, T. M., Arch. Intern. Med. **126**, 1031 (1970).

3. Rowley, D. A., J. Immunol. **64**, 289 (1950).

4. Smith, R. T., Miescher, P. A., and Good, R. A., "The Phylogeny of Immunity," p. 149. Univ. of Florida Press, Gainesville, FL (1966).

5. King, H., and Schumacker, H. B., Jr., Ann. Surg. **136**, 239 (1952).

6. Ellis, E. F., and Smith, R. T., Pediatrics **37**, 111 (1966).

7. Eraklis, A. J., Kevy, S. V., Diamond, L. K., and Gross, R. E., N. Engl. J. Med. **276**, 1225 (1967).

8. Smith, C. H., Erlandson, M., Schulman, I., and Stern, G., Amer. J. Med. **22**, 390 (1957).

9. Laski, B., and MacMillan, A., Pediatrics **24**, 523 (1959).

10. McKinnon, W. M. P., Boley, S. J., and Manpel, J., Amer. J. Dis. Child. **98**, 710 (1959).

11. Robinson, T. W., and Sturgeon, P., Pediatrics **25**, 941 (1960).

12. Walter, L. E., and Chaffin, L., Ann. Surg. **142**, 798 (1955).

13. Doan, C., Bruce, M. D., and Wiseman, K., Proc. Cong. Int. Soc. Hematol., 6th (1956).

14. Carpenter, A. F., Wintrobe, M. M., Fuller, E. A., Haut, A., and Cartwright, G. E., J. Amer. Med. Ass. **171**, 1911 (1959).

15. Miller, E. M., and Hagedorn, A. B., Ann. Surg. **134**, 815 (1951).

16. Motohashi, S., J. Med. Res. **43**, 473 (1922).

17. Rowley, D. A., J. Immunol. **64**, 289 (1950).

18. Rowley, D. A., J. Immunol. **65**, 515 (1950).

19. Leung, L. S. E., Szal, G. J., and Drachman, R. H., personal communication.

20. Kalpaktsoglou, P. K., Yunis, E. J., and Good, R. A., Nature (London) **215**, 633 (1967).

21. Kubai, L., and Auerbach, R., Nature (London) **217**, 460 (1968).

22. Kalpaktsoglou, P. K., and Good, R. A., personal communication.

23. Rothberg, H., and Corallo, L. A, Proc. Soc. Exp. Biol. Med. **100**, 220 (1959).

24. Saslaw, S., and Carlisle, H. N., Antimicrob. Ag. Chemother. **1964**, 635.

25. Saslaw, S., and Carlisle, H. N., Proc. Soc. Exp. Biol. Med. **117**, 420 (1964).

26. Saslaw, S., and Carlisle, H. N., Proc. Soc. Exp. Biol. Med. **123**, 565 (1966).

27. Haller, J. A., Jr., Alessi, L., and Drachman, R., Amer. Surg. **34**, 711 (1968).

28. Maaløe, O., doctoral thesis, Munksgaard, Copenhagen (1946).

29. Cohn, Z. A., and Morse, S. I., J. Exp. Med. **110**, 419 (1959).

30. Hirsch, J. G., and Strauss, B., J. Immunol. **92**, 145 (1964).

31. Taliaferro, W. H., and Taliaferro, L. G., J. Infec. Dis **87**, 37 (1950).

32. Bogart, D. B., Biggar, W. D., and Good, R. A., J. Reticuloendothel. Soc., **11**, 77 (1972).

33. Sutliff, W. D., and Finland, M., J. Exp. Med. **55**, 837 (1932).

34. Heidelberger, M., and Adams, J., J. Exp. Med. **103**, 189 (1956).

35. Heidelberger, M., and Cordoba, F., J. Exp. Med. **104**, 375 (1956).

36. Baker, P. J., and Stashak, P. W., J. Immunol. **103**, 1342 (1969).

37. Michael, J. G., and Rosen, F. S., J. Exp. Med. **118**, 619 (1963).

38. Biggar, W. D., Bogart, D. B., and Good, R. A., unpublished data.

Received Sept. 1, 1971. P.S.E.B.M., 1972, Vol. 139.