JOURNAL OF BACTERIOLOGY
Vol. 87, No. 6, pp. 1457-1461 June, 1964

HOST-PARASITE RELATIONSHIPS AMONG GROUP A STREPTOCOCCI

II. INFLUENCE OF SEX ON THE SUSCEPTIBILITY OF INBRED MICE TOWARD STREPTOCOCCAL INFECTION

DONALD S. WILLOUGHBY[1] AND DENNIS W. WATSON[2]

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota

Received for publication 10 February 1964

ABSTRACT

WILLOUGHBY, DONALD S. (University of Minnesota, Minneapolis), AND DENNIS W. WATSON. Host-parasite relationships among group A streptococci. II. Influence of sex on the susceptibility of inbred mice toward streptococcal infection. J. Bacteriol. **87**:1457-1461. 1964—BALB/Sy mice showed marked sex differences in susceptibility to a strain of *Streptococcus pyogenes* type 18. Castration of the highly susceptible normal adult male increased their resistance 1,000-fold, which approximated that of normal female mice. Treatment of male mice with estrogen and stilbestrol did not affect their resistance significantly. Young, sexually immature male mice, 4 weeks old, were more resistant than mature males. Susceptibility of the resistant females was not affected by age or bilateral ovariectomy, but testosterone injections caused an increase in susceptibility. Treatment of male mice with estrogen and stilbestrol did not modify their resistance. Smears of peritoneal exudates from normal male mice after intraperitoneal injection of streptococci revealed rapid multiplication of the organisms. There was no evidence of multiplication in the resistant normal female and castrated male mice.

Murphy and Watson (1957) showed that the genetic constitution of mice is an important factor in determining whether strains of group A streptococci are virulent or avirulent. BALB/Sy mice were found to be especially susceptible to a strain of *Streptococcus pyogenes* type 18 which had not first been adapted to this host. In contrast, streptococcal strains belonging to types 19 and 28 were avirulent. It was also observed that female BALB/Sy mice were 1,000 times more resistant to type 18 organisms than were the male mice of the same strain.

The present work was undertaken to study factors that might be involved in this sex difference. The effect of bilateral ovariectomy, castration, and varying sex hormone levels on susceptibility of BALB/Sy mice to type 18, group A streptococci is reported.

MATERIALS AND METHODS

Test organism. The strain of group A streptococci used in this work belonged to type 18. It was isolated during an epidemic of acute streptococcal throat infections at Naval Medical Research Unit 4, Great Lakes, Ill., and was extremely virulent for male BALB/Sy mice. The organisms were maintained in the lyophilized state in the presence of defibrinated rabbit blood.

Experimental host. The BALB/Sy mice used were obtained from the mouse colony of the Department of Microbiology, University of Minnesota.

Animal assay. Lyophilized cultures of the test organism were rehydrated in Todd-Hewitt broth and incubated for 18 hr at 37 C. A young 4-hr culture was prepared in the same medium with a 1% inoculum of the 18-hr culture. After diluting this decimally in Todd-Hewitt broth and maintaining at 4 C, 0.5 ml of each dilution was inoculated intraperitoneally into each of five mice. All animals were observed for a period of 10 days, and deaths were recorded. Dead mice were autopsied, and heart blood was streaked on blood-agar plates. Only β-hemolytic streptococci were isolated. The number of organisms contained in each inoculum was determined by plate count. The LD_{50} for each assay was calculated according to the method of Reed and Muench (1938) and was defined as the number of infectious units in 0.5 ml of inoculum which killed 50% of the test animals within 10 days.

[1] Present address: Microbiology Section, Defence Research Board, Suffield Experimental Station, Ralston, Alberta, Canada.

[2] Public Health Service research career program awardee (AI-K6-11651).

Bilateral ovariectomy. Mice were anesthetized with ether and placed, ventral surface down, on a dissecting board. An incision was made through the skin along the vertebral column, and the cut edge of the incision pulled aside to expose the fat pad containing the left or right ovary. After the peritoneal membrane was pierced, the ovary was separated from the fat and the oviduct was cut, thus removing the ovary. The fat pad was then replaced in its original position. The same procedure was used to remove the remaining ovary. On completion, the two cut sections of the skin were jointed by use of Mischel skin clips. These were removed after a period of 1 week.

Castration. Mice were anesthetized with a Nembutal solution (sodium pentobarbital; Abbott Laboratories, North Chicago, Ill.) consisting of absolute alcohol, 10 ml; distilled water, 70 ml; glycerine, 20 ml; and Nembutal (50 mg/ml), 10 ml; 0.01 ml/g of mouse was injected intraperitoneally. In approximately 20 min, the mice were sufficiently anesthetized to proceed with the castration.

The animals were placed on their dorsal surface on a dissecting board, and the scrotum was moistened with 95% alcohol. A small incision was made in the skin, and the testicles were exposed. Both were grasped with a single clamp, and with a clockwise rotation were removed by twisting. With practice, this procedure results in no loss of blood.

After castration, the skin was drawn together by interrupted suture with surgical silk and a curved surgical needle. The animals usually required 1 to 2 hr to recover from the anesthetic. This was aided by keeping them warm under a lamp.

Hormone preparations. The hormones used in this work consisted of testosterone, stilbestrol, and estrogenic substances. Testosterone in the form of testosterone propionate was obtained from The Upjohn Co., Kalamazoo, Mich. Stilbestrol suspended in sesame oil was purchased from the Vitarine Co., Inc., New York, N.Y., and the estrogenic substances consisted of a commercial preparation under the trade name of Amniotin produced by E. R. Squibb and Sons, New York, N.Y.

TABLE 1. *Effect of bilateral ovariectomy on the susceptibility of female BALB/Sy mice toward type 18 cells*

Experimental host	Age	LD_{50}
	weeks	
Ovariectomized female	4	$10^{4.32}$
Ovariectomized female	12	$10^{4.32}$
Normal female	12	$10^{4.16}$
Normal male	12	$10^{0.48}$

TABLE 2. *Influence of testosterone on resistance of female BALB/Sy mice toward infection with type 18 cells*

Experimental host	LD_{50}
Normal female treated with testosterone (10 mg)	$10^{2.89}$
Normal female treated with cottonseed oil	$10^{3.89}$
Normal female	$10^{4.24}$
Normal male	$10^{0.39}$

RESULTS

Bilateral ovariectomy. Ovaries were removed from female BALB/Sy mice of two age groups, 4 and 8 weeks. After 4 weeks, the mice were challenged with type 18 cells. As controls, normal female and normal male BALB/Sy mice, 12 weeks old, were challenged at the same time. The results show that the removal of both ovaries did not increase the susceptibility of female mice toward streptococcal infection (Table 1). Similarly, the age at which the ovaries were removed did not alter susceptibility.

Effect of testosterone injection on the susceptibility of female BALB/Sy mice. Normal female BALB/Sy mice, 12 weeks old, were inoculated with testosterone propionate. Each test animal received 0.1 ml (5 mg) subcutaneously 7 and 4 days prior to intraperitoneal challenge with type 18 cells. The total amount received was thus 10 mg. Since the testosterone was suspended in cottonseed oil, a control group of normal female mice received 0.1 ml of sterile cottonseed oil, subcutaneously, on each of the above days prior to the injection of bacteria. Additional controls for this experiment consisted of normal female and normal male BALB/Sy mice, 12 weeks old, which received only bacteria. Testosterone was found to increase slightly the susceptibility of female BALB/Sy mice toward the type 18 cells (Table 2).

Effect of host age on the susceptibility of BALB/Sy mice. Normal male and female mice of differ-

ent age groups were infected with streptococcal cells to determine whether susceptibility of BALB/Sy mice was related to age. The results (Table 3) show that female mice 4 to 16 weeks old exhibit the same susceptibility to type 18 streptococcal cells. Male BALB/Sy mice of 4 weeks were more resistant to streptococcal infection than were older mice.

Effect of stilbestrol on susceptibility of male BALB/Sy mice. Test animals 12 weeks old were divided into four groups of 10. Group I received 0.5 mg of stilbestrol, subcutaneously, 72 hr before challenge with bacteria. Group II received 1.2 mg, and group III served as controls, receiving only bacterial cells intraperitoneally. Since stilbestrol was suspended in sesame oil, an additional control, group IV, was injected subcutaneously with 0.25 ml of sesame oil 72 hr before inoculation with streptococci. Each mouse received 0.5 ml of Todd-Hewitt broth containing 500 infectious units of type 18 cells. It was found that stilbestrol offered little protection for male BALB/Sy mice (Table 4).

Effect of estrogens on susceptibility. Male BALB/Sy mice, 12 weeks old, were separated into four groups. One group received a total of 3 mg of estrone with 3 weekly injections of 1 mg each, subcutaneously. In the second group, each received 2 mg of estrone with two weekly injections of 1 mg. A third group received 0.4 ml of corn oil, subcutaneously, over a 2-week period, and the fourth group received no treatment prior to challenge with organisms. The virulence tests were conducted in the usual manner 48 hr after the last estrogen injection. Table 5 shows that estrogenic substances had no appreciable effect on increasing the resistance of male BALB/Sy mice toward type 18 cells.

Effect of castration on susceptibility. Male BALB/Sy mice of 8 weeks were challenged with organisms 4 weeks after being castrated. In testing their susceptibility, the usual procedure was followed and they were observed for a 2-week period after inoculation. The castrated animals were more resistant to streptococcal infection than were the normal males (Table 6). The susceptibility of the castrated animals with an LD_{50} of $10^{3.69}$ organisms closely approximated that of normal females.

Peritoneal cell response after animal inoculation. An attempt was made to study the cell response in the peritoneal fluid of the mice after intraperitoneal inoculation with type 18 cells. The cellular response in normal female, normal male, and castrated male BALB/Sy mice was investigated. Male BALB/Sy mice, 8 weeks old, were castrated 4 weeks before challenge with organisms. The normal female and normal male mice were 12 weeks old at the time of inoculation. In each group, 28 mice were used. One-half served as controls and received only organisms, and the

TABLE 3. *Effect of age on the susceptibility of BALB/Sy mice toward Streptococcus pyogenes type 18*

Host	Age	LD_{50}
	weeks	
Female	4	$10^{3.32}$
	6	$10^{3.49}$
	16	$10^{4.16}$
Male	4	$10^{2.95}$
	8	$10^{0.48}$
	12	$10^{0.69}$
	16	$10^{0.18}$

TABLE 4. *Effect of stilbestrol on susceptibility of male BALB/Sy mice toward type 18 cells*

Stilbestrol treatment	Fraction of animals surviving	Per cent mortality
Stilbestrol (0.5 mg)	2/10	80
Stilbestrol (1.2 mg)	3/10	70
Sesame oil (0.25 ml)	0/10	100
Untreated	1/10	90

TABLE 5. *Effect of estrogens on susceptibility of male BALB/Sy mice to type 18 cells*

Experimental host	LD_{50}
Normal male treated with 3 mg of estrogen	$10^{1.43}$
Normal male treated with 2 mg of estrogen	$10^{1.11}$
Normal male treated with corn oil	$10^{0.11}$
Untreated male	$10^{0.58}$

TABLE 6. *Effect of castration on resistance*

Experimental host	LD_{50}
Castrated male	$10^{3.69}$
Normal male	$10^{0.38}$

TABLE 7. *Differential counts on mouse peritoneal fluid after intraperitoneal challenge with type 18 cells**

Time	Normal female		Normal male		Castrated male	
	PMN	Mono	PMN	Mono	PMN	Mono
hr						
1	18	82	34	64	38	62
2	33	67	56	44	49	50
4	82	18	67	33	85	15
6	83	17	70	30	84	16

* Differential counts are expressed as percentages. PMN represents polymorphonuclear leukocytes. Mono represents mononuclear cells.

other half was inoculated with organisms and sacrificed 1, 2, 4, 6, 8, 10, and 18 hr later. Smears were prepared from fluid of the peritoneal cavity according to the standard method of blood film preparation as outlined by Kolmer and Boerner (1945). All mice received 10^3 organisms intraperitoneally. Differential counts were done on each smear made during the first 6 hr. All female and castrated male control animals survived the inoculation, whereas only 1 of 14 normal male controls survived.

Table 7 contains the results of the differential counts. During the initial period, the majority of host cells were mononuclear, whereas after 4 hr multinucleated cells predominated. In smears of exudates from normal female and castrated male mice, no extracellular streptococcal cells were observed, but occasionally organisms were seen within the cytoplasm of mononuclear cells. In contrast, smears from normal male exudates prepared at 2-hr intervals revealed a progressive increase in number of streptococcal cells from 4 hr until 10 hr.

DISCUSSION

In a study of host-parasite relationships, both the virulence attributes of the parasite and the characteristics of the host must be considered. The virulence of group A streptococci is due to the presence of type-specific M protein (Rothbard, 1948; Lancefield, 1954) and, to a lesser degree, to the capsule, consisting of hyaluronic acid (Kass and Seastone, 1944). That the choice of host for host-parasite investigations is important becomes evident when one considers that strains of group A streptococci on primary isolation from human infections are avirulent for mice, even when containing M protein and hyaluronic acid. Only after repeated animal passage do group A streptococci exhibit increased virulence for mice. This is due either to an adaptation of the parasite to the animal tissue or to a selection of more virulent organisms by the host.

The characteristics responsible for the extreme virulence of our type 18 strain are not known. Willoughby, Ginzburg, and Watson (1964) found that this strain produced large quantities of capsular hyaluronic acid at a relatively rapid rate. That it was virulent for the male BALB/Sy mouse without adaptation may indicate a more important role of hyaluronic acid as a virulence attribute than has been shown before for other organisms. Murphy and Watson (1957) found that a strain of type 4, group A streptococci, which produced hyaluronidase and yet contained M protein, was avirulent for male BALB/Sy mice. A stock laboratory strain of type 28 which produced no hyaluronic acid was also avirulent. One cannot assume, however, from these observations that hyaluronic acid is the only factor responsible for type 18 virulence; the rate of production as well as quantity should be considered in determining the relative significance of capsular material in group A streptococcal infections. Hyaluronic acid undoubtedly contributes somewhat to the active multiplication and overwhelming of the host defensive mechanisms observed in normal male BALB/Sy mice.

In spite of several reports demonstrating a protective effect of estrogens and stilbestrol against infectious agents (Foley and Aycock, 1944; Von Haam and Rosenfeld, 1942; Fox, Carroll, and Glacy, 1957; Sprunt, McDearman, and Raper, 1938; Lurie, 1950), these substances offered no significant protection for male BALB/Sy mice against type 18 cells. Ovariectomy resulted in no increased susceptibility in the female mice, but testosterone administration to normal females did increase susceptibility slightly. Castration of male mice, which results in a decrease of the androgen, testosterone, increased resistance of male BALB/Sy mice 1,000-fold. Young, 4-week-old mice were more resistant than older males; this may be a reflection of lower levels of testosterone in sexually immature animals.

The mechanism by which castration alters resistance to type 18 cells is not known. In view of the work of Snell and Nicol (1956) showing the influence of testosterone on the phagocytic activity of macrophages, decreased levels of testos-

terone may increase the phagocytic rate or enhance destruction of the bacterial cells once they have been engulfed. Testosterone has been reported to affect the accumulation of mucopolysaccharides in tissues (Asboe-Hansen, 1958). Castration was found to prevent the formation of hyaluronic acid, and the injection of testosterone was found to reactivate it. Such an effect could play an important role in the spread of infectious particles in the ground substance and connective tissue of the host.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant HTS5360 from the National Heart Institute.

LITERATURE CITED

ASBOE-HANSEN, G. 1958. Hormonal effects on connective tissue. Physiol. Rev. **38**:446–462.

FOLEY, G. E., AND W. L. AYCOCK. 1944. The effect of stilbestrol on experimental streptococcal infection in mice. Endocrinology **35**:139–145.

FOX, L. E., W. R. CARROLL, AND J. GLACY. 1957. Fungistatic actions of estrogens and related compounds. J. Am. Pharm. Assoc. Sci. Ed. **46**: 276–278.

KASS, E. H., AND C. V. SEASTONE. 1944. The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of group A hemolytic streptococci. J. Exptl. Med. **79**:319–330.

KOLMER, J. A., AND F. BOERNER. 1945. Approved laboratory technic, p. 70D. Appleton-Century-Crofts, Inc., New York.

LANCEFIELD, R. C. 1954. Cellular constituents of group A streptococci concerned in antigenicity and virulence, p. 3–18. *In* M. McCarty [ed.], Streptococcal infections. Columbia University Press, New York.

LURIE, M. B. 1950. Mechanisms affecting spread in tuberculosis. Ann. N.Y. Acad. Sci. **52**: 1074–1090.

MURPHY, W. H., JR., AND D. W. WATSON. 1957. Susceptibility of inbred mice to group A streptococcal infection. Proc. Soc. Exptl. Biol. Med. **94**:755–758.

REED, L. J., AND H. MUENCH. 1938. A simple method of estimating 50 per cent endpoints. Am. J. Hyg. **27**:493–497.

ROTHBARD, S. 1948. Protective effect of hyaluronidase and type-specific anti-M serum on experimental group A streptococcus infections in mice. J. Exptl. Med. **88**:325–341.

SNELL, R. S., AND T. NICOL. 1956. Effect of testosterone and androstene 3 *beta*-17 *alpha*-diol on the activity of the reticulo-endothelial system. Nature **178**:1405–1406.

SPRUNT, D. H., S. MCDEARMAN, AND J. RAPER. 1938. Studies on the relationship of the sex hormones to infection. I. The effect of the estrogenic and gonadotropic hormones on vaccinia and the spreading factor. J. Exptl. Med. **67**:159–168.

VON HAAM, E., AND I. ROSENFELD. 1942. The effect of the various sex hormones upon experimental pneumococcus infections in mice. J. Infect. Diseases **70**:243–247.

WILLOUGHBY, D. S., Y. GINZBURG, AND D. W. WATSON. 1964. Host-parasite relationships among group A streptococci. I. Hyaluronic acid production by virulent and avirulent strains. J. Bacteriol. **87**:1452–1456.