JOURNAL OF BACTERIOLOGY
Vol. 87, No. 6, pp. 1452-1456 June, 1964

HOST-PARASITE RELATIONSHIPS AMONG GROUP A STREPTOCOCCI

I. HYALURONIC ACID PRODUCTION BY VIRULENT AND AVIRULENT STRAINS

DONALD S. WILLOUGHBY,[1] YAEL GINZBURG,[2] AND DENNIS W. WATSON[3]

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota

Received for publication 10 February 1964

ABSTRACT

WILLOUGHBY, DONALD S. (University of Minnesota, Minneapolis), YAEL GINZBURG, AND DENNIS W. WATSON. Host-parasite relationships among group A streptococci. I. Hyaluronic acid production by virulent and avirulent strains. J. Bacteriol. 87:1452-1456. 1964.—Intradermal passage of group A streptococcal strains in rabbits is an easy and effective technique for selecting organisms capable of producing increased amounts of hyaluronic acid. Glutamine was required for in vitro hyaluronate production, although the requirement varied with the organism tested. The virulent type 18 cells produced maximal amounts of hyaluronic acid in the presence of 0.01% glutamine compared with 0.1% for the more avirulent type 28; after animal passage, the glutamine requirement for type 28 decreased to 0.01%. Organisms selected by animal passage produced small quantities of hyaluronic acid in the absence of an exogenous source of glutamine. Glucose at a concentration of 1% gave the greatest stimulation for hyaluronate formation. Growth studies indicated similar rates of multiplication for types 18 and 28; yet, the rate of hyaluronate production under these conditions was much greater for the more virulent type 18. Because of these observations, it is suggested that the rate of hyaluronate production is a contributing factor in the virulence of type 18.

Many strains of group A streptococci isolated from nasopharyngeal and throat swabs during streptococcal epidemics produce large, moist, and extremely mucoid colonies (Kuttner and Krumwiede, 1941; Johnson and Furrer, 1958; Kass and Seastone, 1944); this colonial appearance is associated with hyaluronic acid synthesis. Johnson and Furrer (1958) reported that respiratory infectivity may be dependent on hyaluronic acid formation. Exposure of mice to aerosols of group A streptococci showed a direct correlation between aerosol infectivity and ability to produce large quantities of the capsular material in broth. Custod et al. (1960) concluded from aerosol studies that infection of animals by strains of group A streptococci depended on the production of hyaluronic acid for establishing the organisms in the upper respiratory tract because of its antiphagocytic action.

The ability to produce hyaluronic acid is not restricted to particular serological types of group A streptococci, but rather is a characteristic of individual strains (Rosendal and Faber, 1955). The reason for this variation among strains is not known.

The present investigation compares a virulent strain of group A streptococcus implicated in an epidemic with an avirulent strain with respect to quantity and rate of hyaluronic acid synthesis. The effect of animal passage on the glutamine and glucose requirements for hyaluronate synthesis and rate studies are reported.

MATERIALS AND METHODS

Strains of streptococci. Strains of group A streptococci belonging to serological types 18 and 28 were used. Type 18, isolated at Naval Medical Research Unit 4, Great Lakes, Ill., was associated with a high incidence of acute streptococcal throat infections. On primary isolation, it was extremely virulent for the male BALB/Sy mouse (Murphy and Watson, 1957). When broth cultures of this organism were streaked on the surface of rabbit blood-agar and incubated in a 10% carbon dioxide atmosphere, the resulting colonies appeared large, clear, and mucoid. With subsequent animal passage, no change in colonial appearance was observed. The strain of type 28 had been maintained as a stock culture in this laboratory for a number of years and was

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

[2] Present address: Israel Institute for Biological Research, Ness Ziona, Israel.

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

avirulent for mice (Murphy and Watson, 1957). Before animal passage, this strain formed very minute, transparent colonies producing β-hemolysis. After animal passage, the resulting colonies were larger and more mucoid. All organisms were preserved in the lyophilized state.

Animal passage. The method of Prince, Watson, and Cromartie (1951) was used to pass the organisms successively through an animal host. Of a 5-hr Todd-Hewitt broth culture of the organism, 30 ml were injected intradermally into 20 to 30 sites on the shaved ventral surface of an American Dutch rabbit. After 18 hr, the moribund rabbit was killed, and the lesion was removed aseptically. For successive rabbit skin passages, organisms were isolated from the edematous material of the lesion, cultured in broth for 5 hr, and injected intradermally. Lyophilized cultures in defibrinated rabbit blood were prepared from isolates of each lesion and served as the source of working cultures. All freshly removed lesions were frozen at −70 C.

Hyaluronate assay. In determining the ability of streptococci to produce hyaluronate, a "resting cell" technique was used in which cells metabolized but did not reproduce (Lowther and Rogers, 1956). Lyophilized cultures were rehydrated in Todd-Hewitt broth to which dextrose-buffer was added at a concentration of 50 ml per liter of broth. The dextrose-buffer consisted of dextrose, 6%; $NaHCO_3$, 4%; and $Na_2HPO_4 \cdot 12H_2O$, 4%. Each lyophilized culture was dispensed into 10 ml of broth and incubated at 37 C for 18 hr. From this, a fresh 5-hr culture was prepared, and cells obtained by centrifugation at 1,290 × *g* at 4 C were washed three times in a phosphate buffer of pH 7.1. The buffer consisted of 0.067 M sodium β-glycerophosphate, 0.0034 M dibasic sodium phosphate, 0.0022 M monobasic potassium phosphate, and 0.0011 M thioglycolic acid. The washed cells were diluted in buffer to a concentration of 2.0×10^9 cells per ml and dispensed in screw-capped vials. Samples of sterile glucose and L-glutamine solutions were added to each vial, the concentration depending on the individual experiment. Cell suspensions were incubated in a water bath (37 C) for 9 hr. After centrifugation at 4 C, the supernatant fluid was assayed for hyaluronate content according to the method of Tolksdorf et al. (1949). Turbidity measurements were read as optical density in a Coleman Junior spectrophotometer at 580 mμ. From the optical density value, hyaluronate concentration was determined from a standard curve.

RESULTS

Glutamine requirement for hyaluronate production. Lowther and Rogers (1956) demonstrated that L-glutamine served as an excellent nitrogen donor for synthesis of hyaluronic acid by group A streptococci. The response of our virulent and avirulent organisms toward glutamine was studied by incubating cell suspensions in phosphate buffer containing 5% glucose and varying concentrations of glutamine. After the 9-hr incubation period, the supernatant fluid was assayed for hyaluronate. Maximal capsular material was formed by type 18 cells in the presence of 0.01% glutamine (Fig. 1). Type 18 cells, after animal passage, appeared to be less dependent on glutamine; smaller concentrations had a greater stimulatory effect. The stock culture of type 28 produced only small amounts of hyaluronate with a maximum of 0.13 mg at 0.1% glutamine. After animal passage of type 28 cells, there was a fivefold increase in production of capsular material with the optimal glutamine concentration of 0.01%. Both types 18 and 28 cells, after passage, were capable of synthesizing a slight amount of hyaluronate in the absence of glutamine.

Glucose requirement for hyaluronate synthesis. Since glutamine uptake by gram-positive cocci requires an exogenous source of energy (Gale, 1953), hyaluronate production was investigated in the presence of 0.005×10^{-3} M glutamine and varying concentrations of glucose. Types 18 and 28 varied in their response to glucose (Fig. 2).

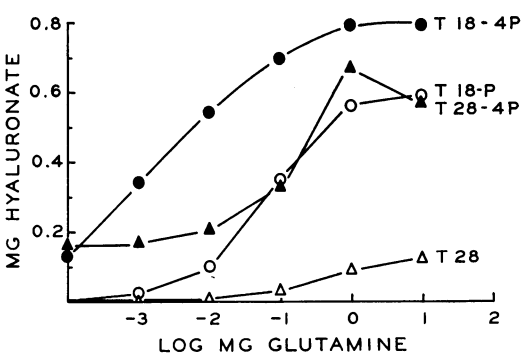

FIG. 1. *Glutamine requirement for hyaluronate production. T18-P and T-28 represent organisms before animal passage; T18-4P and T28-4P were passed four times. Hyaluronate is recorded in milligrams per 10 ml of cell suspension.*

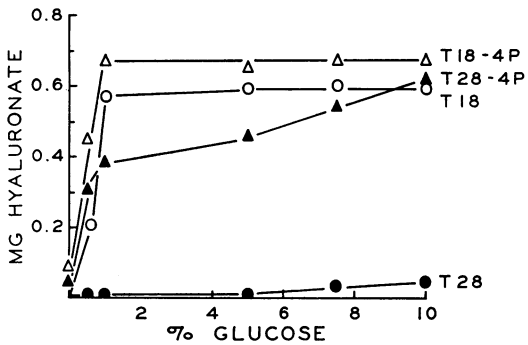

FIG. 2. *Effect of glucose on the production of hyaluronate. T18 cells received one intradermal passage; T28 cells were not passed; T18-4P and T28-4P received four intradermal passages. Hyaluronate was measured as milligrams per 10 ml of cell suspension.*

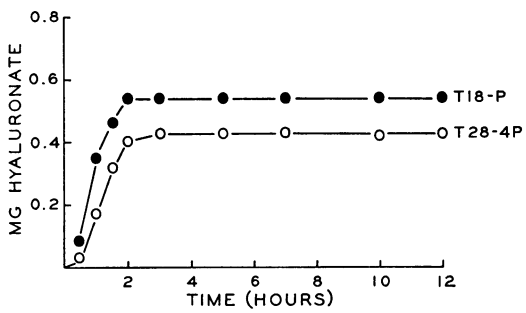

FIG. 3. *Rate of hyaluronate production by type 18 and type 28 cells in maintenance solution. T18-P represents type 18 organisms before intradermal passage, and T28-4P represents type 28 organisms after four passages. Hyaluronate is recorded as milligrams per 10 ml of cell suspension.*

Increasing concentrations of glucose had a negligible effect on hyaluronate production by type 28 cells which had received no animal passages. Type 18 produced a slight amount of capsular material in the presence of 0.5% glucose; increasing the concentration to 1% gave a marked stimulation. Four intradermal passages of type 18 cells resulted in an increased production of hyaluronate at 0.5% glucose with the maximum still produced in the presence of 1%. After animal passage, type 28 organisms produced more hyaluronate as the glucose concentration was increased; the greatest stimulation occurred in the presence of 1%. Higher levels of glucose resulted in slightly increased production of capsular material, with a maximum obtained at 7 to 10%.

Rate of hyaluronic acid production in maintenance solution. Because animal passage of the avirulent type 28 enhanced its hyaluronic acid production and because the optimal concentration of glutamine and glucose had been defined, it was possible to compare the rate of hyaluronic acid production of the avirulent 28 with that of the virulent type 18. This was investigated over a 12-hr period in the following manner. Washed cells were suspended in phosphate buffer containing 0.1% glutamine and 1.0% glucose and dispensed in vials in 10-ml quantities. Vials were placed in a water bath (37 C) and removed at designated time intervals; the suspensions were centrifuged at 4 C, and the supernatant fluid was then assayed for hyaluronic acid. There was little difference between the two cell types in time required to produce their maximal amount of hyaluronate (Fig. 3); both produced their maximal amounts in 2 hr. Type 18 cells, however, within the initial 2 hr not only produced more hyaluronate but at a greater rate.

Growth rate of streptococcal cells in Todd-Hewitt broth. Since type 18 produced more hyaluronate than did type 28 during the initial incubation period in maintenance solution, it was possible that this was a reflection of a higher rate of metabolism of type 18 cells. Growth-rate studies were done in Todd-Hewitt broth to obtain an indication of general metabolic rate. At the same time, hyaluronate formation was followed in this medium.

Lyophilized cultures were rehydrated in broth and grown for 18 hr at 37 C. Each culture was then diluted in broth to give a reading of 41 in a Klett-Summerson photoelectric colorimeter with a red filter (660 mμ). This reading corresponded to a bacterial count of 10^6 cells per ml. A 1-ml amount of the standardized cell suspension was used as inoculum for 50-ml quantities of broth which had been previously warmed to 37 C. The culture tubes were incubated in a water bath (37 C), and the change in turbidity was measured in a Coleman Junior spectrophotometer at a wavelength of 580 mμ. There was essentially no difference in growth rate of the four cultures studied during the 8-hr period (Fig. 4).

At various time intervals, 1 ml was removed from the culture tubes and assayed for hyaluronate content. Type 18 cells, before and after four animal passages, produced hyaluronic acid at a similar rate (Fig. 5). The stock culture of type 28 formed no capsular material in Todd-Hewitt

broth. As found previously, cells of type 28, after animal passage, produced hyaluronic acid and, although the total amount produced at 8 hr approximates that of type 18 cells, it was formed at a reduced rate during the initial 6-hr period.

DISCUSSION

Results of this investigation indicate that intradermal passage of streptococci in rabbits serves as an excellent means of selecting organisms capable of producing increased quantities of capsular material. This was especially evident with our type 28 strain, which produced practically no hyaluronic acid before animal passage. In contrast, passage of the type 18 strain, isolated during an acute streptococcal epidemic, had much less effect on hyaluronate synthesis; it is possible that this strain represents a stable mutant which had already undergone selection in human hosts, whereas passage of type 28 cells resulted in a selection by the experimental animal of organisms capable of producing more capsular material.

The importance of glutamine in the nutrition of β-hemolytic streptococci was demonstrated by McIlwain (1946*a*, *b*). Gale (1953) reported that energy is necessary for the transport of glutamine across cell membranes of nonproliferating gram-positive cocci. Glucose served this purpose effectively. The difference in response to glutamine between type 18 and type 28 cells is given in Fig. 1. After four animal passages, both types produced hyaluronic acid in the absence of an exogenous supply of glutamine. This suggests that such organisms may have a greater internal concentration of glutamine available for hyaluronate synthesis. Before passage, type 28 cells produced hyaluronate only in the presence of high levels of glutamine. After passage, not only was there an increase in hyaluronate synthesis but the organisms appeared to be less dependent on glutamine.

Roseman et al. (1953, 1954) found that D-glucose is incorporated as a unit into both the D-glucosamine and D-glucuronate portions of hyaluronic acid. Figure 2 shows that 1% glucose yields near-maximal quantities of hyaluronate in the presence of glutamine. The increase at higher concentrations of glucose with the strain of type 28 may be due to a stabilizing effect on the synthesizing system (Ginzburg, 1955).

In evaluating the role of hyaluronic acid in group A streptococcal infections, little emphasis has been placed on the rate of its formation. If the significant role of hyaluronic acid lies in its antiphagocytic action (Rothbard, 1948; Foley and Wood, 1959; Custod et al., 1960) and its antibactericidal activity (Skarnes and Watson, 1955), more rapid production of this acid polymer would favor the survival and multiplication of organisms within the host. Evidence presented here showing the more rapid synthesis of hyaluronic acid by type 18 in contrast to the slower rate by the less virulent type 28 may account for its epidemic potential and its great virulence, on primary isolation, toward male BALB/Sy mice.

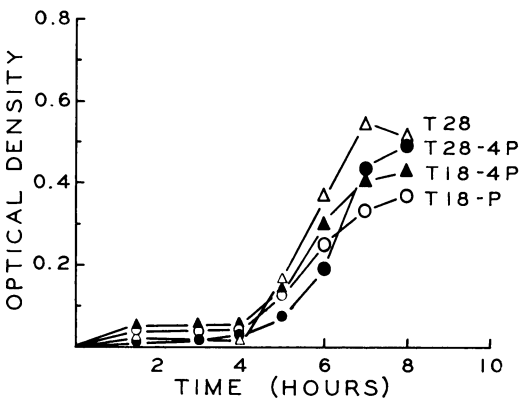

FIG. 4. *Growth rate of streptococcal cells in Todd-Hewitt broth. T28 and T18-P were not passed in animals; T28-4P and T18-4P received four intradermal passages.*

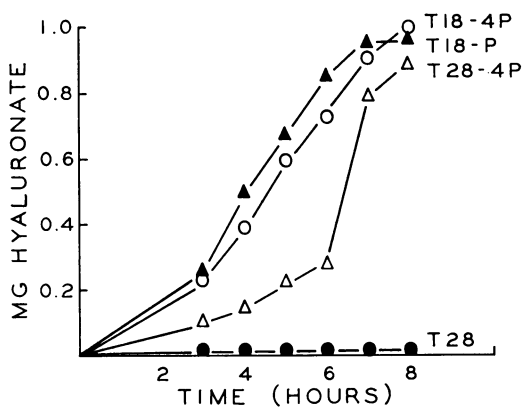

FIG. 5. *Rate of hyaluronic acid production by streptococcal cells in Todd-Hewitt broth. T18-P and T28 were not passed in animals; T18-4P and T28-4P received four intradermal passages. Hyaluronate was measured as milligrams per milliliter of culture.*

ACKNOWLEDGMENT

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

LITERATURE CITED

CUSTOD, J. T., R. I. LYTLE, B. H. JOHNSON, AND P. F. FRANK. 1960. Interdependence of hyaluronic acid and M protein in streptococcal aerosol infections in mice. Proc. Soc. Exptl. Biol. Med. **103**:751–753.

FOLEY, M. J., AND W. B. WOOD. 1959. Studies on the pathogenicity of group A streptococci. II. The antiphagocytic effects of the M protein and the capsular gel. J. Exptl. Med. **110**:617–628.

GALE, E. F. 1953. Assimilation of amino acids by gram-positive bacteria and some actions of antibiotics thereon. Advan. Protein Chem. **8**:285–391.

GINZBURG, Y. 1955. The biosynthesis of hyaluronate by group A streptococci. Ph.D. Thesis, University of Minnesota, Minneapolis.

JOHNSON, B. H., AND W. FURRER. 1958. Hyaluronic acid: a required component of *beta*-hemolytic streptococci for infecting mice by aerosol. J. Infect. Diseases **103**:135–141.

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.

KUTTNER, A. G., AND E. KRUMWIEDE. 1941. Observations on the effect of streptococcal upper respiratory infections on rheumatic children: a three year study. J. Clin. Invest. **20**:273–287.

LOWTHER, D. A., AND H. J. ROGERS. 1956. The role of glutamine in the biosynthesis of hyaluronate by streptococcal suspensions. Biochem. J. **62**:304–314.

MCILWAIN, H. 1946*a*. The metabolism and functioning of vitamin-like compounds. I. Ammonia formation from glutamine by haemolytic streptococci; its reciprocal connexion with glycolysis. Biochem. J. **40**:67–78.

MCILWAIN, H. 1946*b*. The metabolism and functioning of vitamin-like compounds. III. Products of the decomposition of glutamine during streptococcal glycolysis. Biochem. J. **40**:460–464.

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.

PRINCE, J. T., D. W. WATSON, AND W. J. CROMARTIE. 1951. The production in vivo of phase variation among group A streptococci. Bacteriol. Proc., p. 117–118.

ROSEMAN, S., F. E. MOSES, J. LUDOWIEG, AND A. DORFMAN. 1953. The biosynthesis of hyaluronic acid by group A streptococcus. I. Utilization of 1-C^{14}-glucose. J. Biol. Chem. **203**: 213–225.

ROSEMAN, S., J. LUDOWIEG, F. E. MOSES, AND A. DORFMAN. 1954. The biosynthesis of hyaluronic acid by group A streptococcus. II. Origin of the glucuronic acid. J. Biol. Chem. **206**:665–669.

ROSENDAL, K., AND V. FABER. 1955. Streptococcal hyaluronidase. III. The effect of penicillin on the production of hyaluronic acid and hyaluronidase by hemolytic streptococci (Type 24, group A). Acta Pathol. Microbiol. Scand. **36**:263–273.

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.

SKARNES, R. C., AND D. W. WATSON. 1955. The inhibition of lysozyme by acidic polymers from pathogenic bacteria. J. Bacteriol. **70**: 110–112.

TOLKSDORF, S., M. H. MCCREADY, D. R. MCCULLAGH, AND E. SCHWENK. 1949. The turbidimetric assay of hyaluronidase. J. Lab. Clin. Med. **34**:74–89.