

Bacterial Interference

IV. Epidemiological Determinants of the Antagonistic Activity of the Normal Throat Flora Against Group A Streptococci

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A study was performed to identify epidemiological factors such as age, race, sex, and time of culture that might influence the ability of the normal pharyngeal flora to interfere with growth of group A streptococci. From March 1974 through February 1975, throat swabs were obtained from 952 individuals. Cultures were assayed by an agar overlay procedure for the presence of bacteria capable of inhibiting growth of group A streptococci. The observed inhibition was then determined to be bacteriostatic or bactericidal by use of a broth filtrate technique. Regardless of age, race, or sex, subjects were more likely to harbor interfering flora if cultured during the months of March and April, which coincided with the highest prevalence of group A streptococci in the community. Race and sex of subjects appeared not to influence the inhibitory activity of throat flora either quantitatively or qualitatively. However, among individuals with interfering flora, the prevalence of bactericidal organisms increased and bacteriostatic organisms decreased with advancing age. Since the presence of bactericidal, and not bacteriostatic, organisms has been associated with resistance to colonization of the throat by group A streptococci, this higher prevalence of bactericidal organisms in older individuals suggests that bacterial interference may be one of the mechanisms that account for the greater resistance of adults than children to streptococcal throat infection.

Previous studies in this laboratory have revealed the following: (i) certain constituents of the normal pharyngeal flora inhibit growth of group A streptococci in vitro, and this inhibition may be bacteriostatic or bactericidal; (ii) bactericidal inhibitory flora are more prevalent in the throats of uninfected children than in children infected with group A streptococci; (iii) children with a bactericidal flora are less likely to acquire group A streptococci in their throats after exposure to epidemic strains than are children with a bacteriostatic or noninhibitory flora; and (iv) among children who become colonized with group A streptococci and are not treated with antibiotics, cultures obtained after the colonization contain more inhibitory organisms than cultures taken before or during the colonization (1, 5). This latter observation suggests that group A streptococci may exert a selective pressure that favors emergence of inhibitory strains in the resident flora. It was thus hypothesized that repeated transient exposures to group A streptococci throughout early life may select for a progressively more inhibitory flora and that this may be one of the mechanisms whereby adults ultimately become more resistant than children to streptococcal

infection of the pharynx. Since previous studies had been conducted exclusively in children, the present investigation was designed to evaluate the influence of age and other epidemiological factors upon the quantitative and qualitative aspects of inhibitory throat flora.

MATERIALS AND METHODS

Study population. During one morning each week from 1 March 1974 to 28 February 1975, throat swabs were obtained from all patients and their parents, siblings, and friends who reported to the Pediatric Outpatient Clinic of the Creighton Memorial St. Josephs Hospital, Omaha, Neb. Informed consent was obtained for each individual. The population studied was drawn primarily from the metropolitan area of Omaha, including surrounding Douglas County. A wide range of ethnic and socioeconomic groups were represented, with a slight predominance of individuals of lower socioeconomic status. Most patients cultured had come to the clinic for immunization, preschool physical examinations, allergies, or orthopedic problems; few suffered infectious diseases.

Three criteria were established for admission to the study. They were: (i) absence of group A streptococci on culture, (ii) lack of antimicrobial therapy in the week preceding the culture, and (iii) not having been included in the study previously. Of 952 indi-

viduals cultured, 710 met these criteria. For analysis of data, individuals were separated into three age groups: (i) preschool, 0 to 4 years; (ii) school age, 5 to 15 years; and (iii) adult, over 15 years. The ages of those included in the study ranged from 8 days to 67 years. Other groupings were also made on the basis of sex, race, and month at time of culture. All tests for statistical significance were performed by chi-square analysis with Yate's correction factor.

Throat swabs. Throat swabs were obtained with a dry, sterile, cotton-tipped swab pressed firmly on the tonsillar pillars, around Waldeyer's ring and in a crisscross pattern over the posterior pharyngeal wall. All swabs were immediately placed in 2 ml of brain heart infusion broth (Baltimore Biological Laboratories) and used to inoculate cultures and for interference assays within 1 h.

Throat cultures. The swab-containing broth was shaken vigorously for 3 min. Then 0.01 ml of the broth was placed on the surface of a 5% sheep blood agar plate and streaked for isolation of colonies in the four-quadrant fashion. After incubation for 24 h at 37°C in 10% CO₂ in air, cultures were examined for the presence of beta-hemolytic streptococci. Among these, group A streptococci were identified by their susceptibility to bacitracin (A disk, Baltimore Biological Laboratories).

Interference assays. Both procedures used in this study have been described in detail previously (1). An agar overlay technique was used for screening throat flora for inhibitory activity against group A streptococci. A 1:500 saline dilution was made of the swab-containing broth. Two milliliters of this dilution was placed on the surface of a brain heart infusion agar plate, and the excess was siphoned off. After overnight incubation, this plate was replicated onto a sheep blood agar plate and overlaid with brain heart infusion agar containing 20% sheep blood. Group A streptococci were then inoculated onto the overlay surface. After a second overnight incubation, interference was detected as areas on the overlay surface where growth of the group A streptococcus and hemolysis had been inhibited. An assay was considered positive if at least one such area was present. Colonies of inhibitory flora present in corresponding areas on the replicate sheep blood agar plate were isolated, identified, and tested further to determine the nature of the inhibitory activity by a broth filtrate technique (1). No more than one isolate of each morphologically different interfering organism on a single overlay assay was tested by this second procedure. After each of these isolates had been incubated for 72 h in Trypticase soy broth (Baltimore Biological Laboratories), all bacteria were removed by microfiltration (0.45- μ m filter, Nalge Co.). Group A streptococci were inoculated into this filtrate to yield a final population of approximately 10⁴ colony-forming units/ml. After the filtrates were incubated overnight, they were examined for macroscopic growth, and 0.01 ml was subcultured onto a sheep blood agar plate. Each filtrate containing fewer than 10 colonies on subculture was considered to be bactericidal. Filtrates showing no macroscopic growth but yielding more than 10 colonies were considered bacteriostatic. Fil-

trates showing macroscopic growth were recorded as non-inhibitory.

All interference assays were incubated at 37°C in 10% CO₂ in air. Therefore, only aerobic and facultative constituents of the throat flora were evaluated. Individuals reading the results of each test were unaware of the source of the culture. The test organism used in all assays was a clinical isolate (*Streptococcus pyogenes*, Lancefield group A, M-type 12, T-type 12) that has been shown to respond to inhibition by the normal flora in a manner identical to that of other group A streptococci of varying M- and T-types (1, 5).

RESULTS

Occurrence of interfering flora. Results from the overlay technique were first analyzed on the basis of month at time of culture (Fig. 1). The percentages of assays positive for interference for March and April were each significantly higher than that observed during each of the other 10 months of the study year (May 1974–February 1975, $P < 0.025$). To examine the prevalence of group A streptococci in the community from which the uninfected study population was drawn, results were obtained from 32,278 throat cultures that had been processed during the study year by the Omaha-Douglas County Public Health Department Rheumatic Fever Screening Program. When the percentage of these throat cultures positive for group A streptococci was determined for each month of the study year, the highest percentage was found to have occurred during March and April (Fig. 1). Data for the 2 months (January and February 1974) preceding the study year are also shown to indicate that the high prevalence in March and April represented a true peak.

Neither sex, race, nor age significantly influenced the occurrence of interfering flora. Of 277 males tested, 159 (57%) had assays positive for

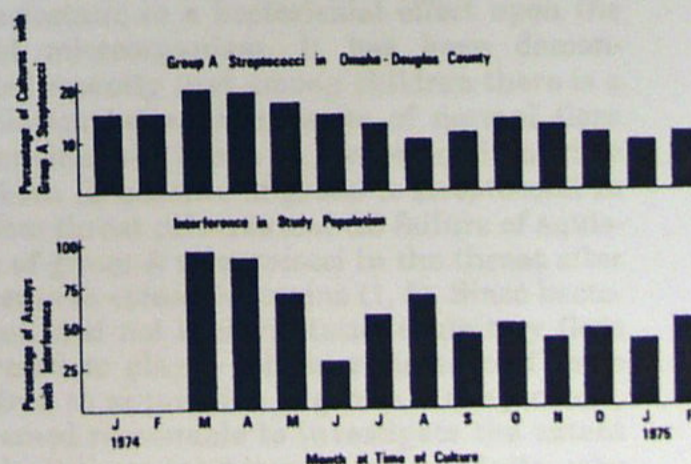


FIG. 1. Prevalence of interfering flora in the study population and group A streptococci in Omaha-Douglas County.

interference, whereas 253 of 433 (58%) females had positive assays. Among the two major races represented, 286 of 474 (60%) whites had positive assays whereas 106 of 197 (54%) blacks had positive assays. When the study population was grouped by age, the percentages of assays positive for interference were 53% (79 of 150), 61% (114 of 187), and 59% (219 of 373) for the preschool, school age, and adult groups, respectively.

Qualitative variations in interfering flora. Since individuals in each of the three groups were equally likely to harbor interfering flora, the microorganisms responsible and the qualitative nature of this interference were examined. For these analyses, only data from individuals with positive assays for interference were used. Several age-dependent differences were found (Table 1). The percentage of positive assays containing interfering nonhemolytic streptococci increased significantly from the preschool to the adult group, whereas the percentage of positive assays containing interfering alpha-hemolytic streptococci decreased significantly from the preschool to the adult group. The number of other interfering organisms (primarily neisseriae and micrococci) was insufficient to allow meaningful comparisons between age groups.

The qualitative nature of the inhibitory activity was compared among the three age groups by analysis of data from the broth filtrate assays. Since alpha- and nonhemolytic streptococci represented the large majority of all interfering organisms, only results of assays performed with these two organisms were considered. The percentage of these isolates that demonstrated bactericidal activity was significantly higher in the adult than in the preschool

or school age groups, or both (Table 2). Reciprocally, the percentage of isolates from the adult group showing bacteriostatic activity was significantly lower than that of the preschool group or the combined groups of children 15 years of age and under. When results were analyzed for each of the two types of streptococci, a similar trend was observed (Table 2). For both alpha- and nonhemolytic streptococci, the percentage of bactericidal isolates increased and the percentage of bacteriostatic isolates decreased with progressive increments in age. The differences in the nature of inhibitory activity of alpha-hemolytic streptococci between the preschool (or the combined preschool and school age) and adult groups were significant (Table 2). The same trend was observed with the nonhemolytic streptococci; however, the differences were not statistically significant. No other qualitative variations in the interfering flora were detected in the study population.

DISCUSSION

This study detected significant differences between children and adults in the qualitative nature of their normal throat flora and provided further insight into the phenomenon of bacterial interference against group A streptococci. Casual inspection of aerobic and facultative bacteria on throat cultures from healthy children and adults reveals a remarkable uniformity in both numbers of organisms and genera represented. However, results of this and earlier studies have indicated that strains of pharyngeal bacteria identical in morphological, serological, and biochemical characteristics often differ widely in their interaction with other microorganisms in vitro (2, 3; W.E. Sanders, Jr., *Microbiology-1975*, p. 106-109, American Society for Microbiology, Washington, D.C., 1975). Some may interact indifferently. Others may interact antagonistically, with either a bacteriostatic or a bactericidal effect upon the target microorganism. It has been demonstrated recently that among children there is a correlation between presence of normal flora bactericidal for group A streptococci in vitro and both (i) absence of group A streptococci in random throat cultures and (ii) failure of acquisition of group A streptococci in the throat after exposure to epidemic strains (1, 5). Since bactericidal, and not bacteriostatic, inhibitory flora appeared to play a role in resistance of some children to acquisition of group A streptococci, it seemed reasonable to investigate the extent to which these inhibitors occur in adults, who are known generally to be more resistant to streptococcal infection of the pharynx.

Profound differences were identified in com-

TABLE 1. Composition of the interfering flora of the study population grouped by age

Group	No. of positive assays ^a	No. (%) with interfering non-hemolytic streptococci	No. (%) with interfering alpha-hemolytic streptococci
Preschool, 0-4 yr	79	68 (86)	65 (82)
School age, 5-15 yr	114	102 (90)	86 (75)
Adult, over 15 yr	219	207 (95) ^b	141 (64) ^c

^a At least one area of interference on the agar overlay assay.

^b Significantly higher than preschool ($\chi^2 = 4.69$, $P < 0.05$) and preschool plus school age ($\chi^2 = 4.67$, $P < 0.05$).

^c Significantly lower than preschool ($\chi^2 = 7.87$, $P < 0.005$) and preschool plus school age ($\chi^2 = 8.88$, $P < 0.005$).

TABLE 2. Broth filtrate activity of interfering isolates from the study population grouped by age

Group	Streptococcal isolates tested			Alpha-hemolytic streptococci tested			Nonhemolytic streptococci tested		
	Total	No. (%) bactericidal	No. (%) bacteriostatic	Total	No. (%) bactericidal	No. (%) bacteriostatic	Total	No. (%) bactericidal	No. (%) bacteriostatic
Preschool, 0-4 yr	92	37 (41)	50 (54)	46	15 (33)	30 (65)	46	22 (48)	20 (44)
School age, 5-15 yr	134	65 (49)	62 (46)	64	28 (44)	33 (52)	70	37 (53)	29 (41)
Adult, over 15 yr	167	104 (62) ^a	58 (35) ^b	78	47 (60) ^c	28 (36) ^d	89	57 (64)	30 (34)

^a Significantly higher than school age ($\chi^2 = 5.17, P < 0.02$), preschool ($\chi^2 = 10.75, P < 0.002$), and school age plus preschool ($\chi^2 = 10.636, P < 0.002$).

^b Significantly lower than preschool ($\chi^2 = 8.59, P < 0.005$) and preschool plus school age ($\chi^2 = 8.01, P < 0.005$).

^c Significantly higher than preschool ($\chi^2 = 7.76, P < 0.008$) and preschool plus school age ($\chi^2 = 7.37, P < 0.008$).

^d Significantly lower than preschool ($\chi^2 = 8.83, P < 0.005$) and preschool plus school age ($\chi^2 = 7.52, P < 0.008$).

parisons between adults and children. Although approximately one-half of each of the preschool, school age, and adult groups harbored flora that inhibited group A streptococci in vitro, the organisms responsible for the inhibition and the qualitative nature of the antagonism differed markedly. The adults harbored a significantly greater percentage of interfering nonhemolytic streptococci and a significantly lower percentage of interfering alpha-hemolytic streptococci than children 15 years of age and under. In addition, the adult flora contained significantly more bactericidal inhibitors and significantly fewer bacteriostatic inhibitors than the childhood flora.

Upon examination of the interfering flora across the spectrum of age, it appeared as if the flora of school age children were in transition from that of the preschool children toward that of the adult group. The interfering flora in the school age group contained more nonhemolytic streptococci and less alpha-hemolytic streptococci than that of the preschool group. In addition, the interfering flora of the school age children contained more bactericidal inhibitors than the flora of the preschool children.

Proof of the occurrence of a gradual transition from a predominantly bacteriostatic to a predominantly bactericidal interfering flora with advancing age of individuals, of course, must await longitudinal studies that span at least 16 years. However, two additional observations support this contention. First, in an earlier study, it was shown that children who developed asymptomatic pharyngeal infection with group A streptococci harbored more inhibitory flora in cultures obtained after infection than in cultures obtained before or during the infection (1). Second, during this study, interfering flora was detected significantly

more frequently among individuals cultured in March and April than in any other month; and this coincided with the 2 months of highest prevalence of group A streptococci in the community from which our study subjects were drawn. Both of these observations suggest that transient exposure to group A streptococci exerts a selective pressure that favors emergence of inhibitory strains in some individuals. If so, repetitive exposures might be expected to result ultimately in selection of the bactericidal inhibitors that in this study were shown to predominate among adults. Conversely, systemic antibacterial therapy might be expected to delay or reverse this evolution of a more inhibitory flora. This possibility received strong support in an earlier study in which a 7-day course of orally administered penicillin V was shown to markedly diminish or eliminate inhibitors from the throats of young adults (4). In addition, most of the inhibitory nonhemolytic streptococci in the subjects were replaced by alpha-hemolytic streptococci and these changes persisted for the duration of the period of observation (at least 3 weeks) after treatment. In other words, therapy with penicillin appeared to have reverted the characteristics of the flora in these subjects from those of adulthood toward those of childhood.

In summary, the interfering throat flora of adults has been shown to contain more nonhemolytic streptococci and more bactericidal inhibitors of group A streptococci than the flora of children. Since bactericidal flora has been associated with resistance to acquisition of group A streptococci in the pharynx, this may in part explain the greater resistance of adults than children to streptococcal infection. Also, the data suggest indirectly that a bactericidal inhibitory flora may evolve slowly with age and

that this process may be facilitated by exposure to group A streptococci.

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