Production of Bacteriocin-Like Inhibitors by Group A Streptococci of Nephritogenic M Types

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Application of a bacteriocin-typing scheme that had been designed to minimize the inhibitory effects of hydrogen peroxide and acidic metabolites demonstrated a high incidence (72%) of bacteriocin-like inhibitors belonging to 5 different production (P) types in 61 strains of group A streptococci isolated in association with the development of acute glomerulonephritis. By contrast, only 4 of 16 (25%) rheumatic fever-associated strains were inhibitor positive, and 3 of these strains were P type 604, a P type not detected in any of the nephritis isolates. The P type designation was found to be identical for all inhibitor-positive strains within each particular M serotype, regardless of whether the source was a nephritis patient or an individual having an uncomplicated streptococcal infection. The incidence of inhibitor-positive strains was particularly high in strains of M types 2, 4, 12, 25, 57, and 60; it was moderately high in M types 49, 52, and 55 and absent in M1 and M3. Although the results indicate that the bacteriocin-like inhibitors detected in this study are probably unlikely to have a direct pathogenetic role in either rheumatic fever or acute glomerulonephritis, it does seem that the present P-typing scheme is a useful epidemiological tool, particularly for the presumptive identification of and differentiation among group A streptococcus strains of nephritogenic serotypes.

The M proteins, in addition to being antiphagocytic virulence factors in acute group A streptococcus infections (8), also have an important practical application as serotype-specific strain markers in epidemiological investigations. Although the role of the M proteins in the pathogenesis of the delayed sequelae to group A streptococcus infections is not yet clear, it has been established that strains of certain M types are more commonly implicated in the genesis of either rheumatic fever (M type 5) or nephritis (M types 1, 2, 3, 4, 12, 25, 49, 52, 55, 57, 60) (2, 10).

Most of the known group A streptococcus cellular components and extracellular products have now been systematically evaluated for their pathogenetic potential. Kuttner (4) reported that streptococcus strains of the nephritogenic serotypes 4, 12, and 49 produced bacteriocin-like inhibitory activity more frequently than strains of nonnephritogenic serotypes. By contrast, other investigators were unable to demonstrate any significant difference in the inhibitory activity of nephritis-associated and nonnephritis-associated strains (6, 7). In other studies (1, 9), it seemed that isolates from rheumatic fever cases had a higher incidence of inhibitor production than strains from uncomplicated pharyngitis. However, no characterization of the "bacteriocin-like" inhibitory agents was reported, and it is now evident that the test conditions used in all of these studies would not have excluded inhibition due to either hydrogen peroxide (5, 6) or acidic metabolites (17).

A bacteriocin "fingerprinting" scheme has been developed for hemolytic streptococci, based upon the evaluation of both the inhibitor-producing activity (P type) and inhibitor sensitivity (S type) of the test strains (14). Application of this scheme enabled the differentiation of 350 group A streptococci into 13 P types, but with over 70% of the strains being either P type 004 or 204 (14). In other studies, it has been demonstrated that the production of inhibitory levels of

acidic metabolites can account for the production of these particular P type patterns and that this nonbacteriocin-related activity can be eliminated by appropriate buffering of the test medium (17).

Previous investigations have shown that all of the tested strains of M type 4 and M type 57 group A streptococci are P type 655 and P type 614, respectively, and that these inhibitory patterns are not produced by strains of any other M types (14). Characterization studies subsequently demonstrated that these strains were producing proteinaceous antibiotics of a bacteriocin-like nature (3, 13).

In the present study, the previously described P-typing scheme was modified to enable the detection of bacteriocin-like activity by group A streptococcus strains under conditions intended to exclude nonspecific inhibition due to either hydrogen peroxide or acidic metabolites. With this scheme, various group A streptococci have been tested for bacteriocinogenicity in an attempt to give some further insight into the possible relationship of this property to the M type and to the rheumatogenic or nephritogenic potential of the bacteria.

MATERIALS AND METHODS

Bacterial strains. The previously described set of nine indicator strains (11-19) was used for P typing (14). A single representative strain of each of the recognized M types of group A streptococci (M1-M81) was obtained from the late L. W. Wannamaker. Types 7, 10, 16, 20, 21, 35, 44, and 45 were not included because they are not group A streptococci (types 7, 16, 20, 21) or are duplicate numbers assigned to other M types (10 = 12, 35 = 49, 45 = 24, 44 = non-M producer). Strains used in this study were isolated from patients having epidemiologically distinct symptomatic infections and were generously made available by the following investigators: A. L. Bisno, P. Ferrieri, C. A. M. Fraser, D. R. Martin, G. E. Meekin, E. V. Potter, D. S. Selinger, S. Skjold, L. W. Wannamaker, and J. B. Zabriskie.

P-typing test. The procedure used for P typing was essentially as previously reported (14) and may be summarized as

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TABLE 1. P type designations of inhibitor-positive M prototype strains of group A streptococci

P type ^a	M type strain no.
777	2, 11, 12, 25, 28, 60, 66, 67, 71, 76
674	34, 39, 42
655	4
626	55
614	57, 69
216	61, 64
214	15, 63

^a Under conditions of anaerobic incubation, except for the prototype strains of M types 34, 39, and 55, for which aerobic incubation gave optimal inhibitor production.

follows. The typing medium was Columbia blood agar base (Difco Laboratories) supplemented with 5% (vol/vol) human blood and (for the present study) 0.5% (wt/vol) calcium carbonate and was poured as a layer (20 ml) on a base of saline agar (20 ml) in 9-cm glass petri dishes. As an additional measure to eliminate inhibition due to hydrogen peroxide, the typing medium was, for some special tests, supplemented with 1,000 U of freshly prepared catalase per ml (from bovine liver; Sigma Chemical Co.). The test strains were propagated on blood agar. A 1-cm-wide streak of the potentially inhibitory strain was inoculated across the diameter of the surface of the typing medium by means of a cotton swab, and the plate was incubated at 32°C for 18 h. For each test strain, one plate was incubated aerobically and another was incubated anaerobically. Then, the visible growth was removed by scraping with the edge of a glass slide, and the plate was inverted for 30 min over a circle of chloroform-soaked filter paper in the lid of the petri dish. After the surface of the plate had been exposed to the air for 15 min, Todd-Hewitt broth (Difco) cultures (18 h, 37°C) of the nine indicator strains were streaked with cotton swabs at right angles to the line of the original growth of the test strain. After incubation at 32°C for 18 h, inhibition of growth of the indicator strains was recorded as + (definite inhibition), (+) (partial inhibition), or - (no inhibition). Strains giving (+) reactions were retested. If (+) reactions were consistently produced, they were considered to be positive reactions and were recorded as such in the P type score. The inhibition patterns were recorded in code form by considering the nine indicators as three triplets (e.g., I1, I2, I3; I4, I5, I6; I7, I8, I9). Positive reactions against each indicator were given a score of 4, 2, or 1 depending on whether the indicator was, respectively, the first, second, or third member of the triplet. Negative reactions were scored as zero. The total score for each triplet thus specified uniquely the reactions against the three indicators. The complete P type code was written as a sequence of three numbers, consecutively defining the reactions within the three triplets. Unless otherwise indicated, the P type designations reported in the present study are those obtained by anaerobic incubation of the test strains.

RESULTS

Examination of the M prototype group A streptococci showed that 21 of the 73 strains (29%) were producers of bacteriocin-like inhibition, with seven different P types identified (Table 1). The production of inhibitory activity by the M34, M39, and M55 strains was greater under aerobic incubation conditions. The increased activity also occurred on catalase-supplemented typing medium, indicating that it was not attributable to hydrogen peroxide. Inhibitor production by the other strains, particularly M4, M11, M12, M25,

M28, M57, and M71, was favored by anaerobic incubation.

The incidence of inhibitor production in the set of the rheumatic fever-associated strains was 25% (Table 2). Three of the four inhibitor-producing strains were P type 604. Four of the noninhibitory strains were M type 5. Another 14 M type 5 isolates from patients not having rheumatic sequelae were also tested and found to be inhibitor negative.

A collection of 61 strains implicated in independent episodes of nephritis and belonging to commonly encountered nephritogenic serotypes was P typed; 72% were inhibitor positive, with five P types detected (Table 3). Another 113 isolates, not involved in the development of nephritis but belonging to the common nephritogenic M types, were tested; 63% were inhibitor positive (Table 3). The P type detected was consistent for all of the inhibitory strains of each particular M type, and none of the inhibition patterns seemed to be particularly prevalent in the nephritis-associated isolates.

DISCUSSION

The inhibitor P-typing scheme used in the present study is a modification of that previously described (14), in that calcium carbonate was added to the typing medium to minimize the creation of inhibitory pH levels due to production of acidic metabolites by the test strains. Appropriate buffering of the test medium becomes particularly important with use of anaerobic incubation conditions, since this markedly increases acid production (17) in addition to its effect of enhancing the yield of some group A streptococcus bacteriocin-like substances (14). Anaerobic incubation of the test strains, when combined with increased buffering capacity of the medium, has made it possible to effectively eliminate nonspecific inhibition of the indicator organisms by either hydrogen peroxide or acidic by-products of metabolism. Since some strains appear to produce bacteriocin-like inhibitors more reliably during aerobic growth, it is preferable to incubate two sets of test cultures for P typing, one aerobic and the other anaerobic.

In the current study, it was found that 29% of the M protein prototype strains were inhibitor positive, with almost half being P type 777. Although a previous survey had indicated that P type 614 may have been a unique characteristic of M type 57 group A streptococci (13), the availability of additional M prototypes in the present investigation has made it possible to show production of this same P type by

TABLE 2. Relationship of P type to serotype in rheumatic fever-associated group A streptococci

Strain designation	Source	Serotype	P type
GT6521	L. W. Wannamaker	M5	000
72-469	L. W. Wannamaker	M5	000
72-500	L. W. Wannamaker	M5	000
Manfredo	A. L. Bisno	M5	000
8002900	E. V. Potter	M6	777
GT6527	L. W. Wannamaker	M14	000
GT8246	L. W. Wannamaker	M18	000
GT6525	L. W. Wannamaker	M24	000
Vaughn	A. L. Bisno	M24	000
EP29	E. V. Potter	M29	000
8005200	E. V. Potter	M41	604
8102200	E. V. Potter	M53	000
8101500	E. V. Potter	M53	000
36A	D. S. Selinger	T13B3264	000
39G	D. S. Selinger	T22	604
39N	D. S. Selinger	T12	604

TABLE 3. Bacteriocin-like inhibitor production by nephritis-associated and nephritis-nonassociated group A streptococci of nephritogenic M types

M type	P type	Inhibitor production by strains	
		Nephritis associated	Nonnephritis associated
1	000	3	7
2	777	7	6
2	000	0	1
3	000	1	6
4	655	2	14
12	777	12	15
12	000	2	3
25	777	0	7
25	000	0	1
49	476	6	5
49	000	7	14
52	476	1	3
52	000	1	3
55	626 ^a	2	4
55	000	3	4
57	614	7	12
60	777	7	5
60	000	0	3

^a P type pattern obtained by aerobic incubation of the test strains.

the M type 69 prototype. Bacteriocin production by M type 57 strains has been found to be under the control of a 2.2-megadalton plasmid (12), and what appears to be an identical plasmid is also present in the prototype M69 strain (W. J. Simpson and J. R. Tagg, unpublished data).

Some years ago, the hypothesis was advanced that rheumatic carditis might result from the combined cardiotoxicity of heart-reactive antibodies and group A streptococcus bacteriocins (15). In support of this, some investigators reported that the incidence of bacteriocin-like activity may be particularly high in rheumatic fever-associated strains (1, 9). Although in the current study bacteriocinogenicity was not a common property of rheumatogenic strains, it is perhaps noteworthy that three rheumatic fever isolates were P type 604, a type not recorded for any of the other group A streptococcus strains examined in either the present or previous (14) surveys.

Strains of many of the common nephritogenic M types, particularly 2, 4, 12, 25, 57, and 60, had a high incidence of inhibitor production, irrespective of whether the strains had actually been isolated in association with the development of nephritis. An interesting feature was the absolute uniformity of P type detected among the inhibitor-positive isolates of each particular M type.

Inhibitor production occurred somewhat less frequently in strains of M types 49, 52, and 55. The inhibitor-positive M49 and M52 strains were P type 476, a pattern characteristic of streptococci A-FF22, the first of the group A streptococcus bacteriocins to be characterized (14, 16). It is possible that more detailed examination of pairs of strains of differing M types that share a common P type designation could yield data regarding the possible origin of the diversity of the M proteins.

With use of the present scheme, there was no evidence for production of bacteriocin-like substances by the available strains of serotypes M1 and M3. However, it is certainly feasible that the appropriate indicators may not yet be available for the detection of the full repertoire of streptococcal bacteriocins or that the particular test conditions used

may be inappropriate for the expression of bacteriocinogenicity by some of the test strains.

It does seem that testing for bacteriocin-like activity could provide yet another useful index (in addition to M typing) for the detection of and differentiation among streptococci having so-called "nephritogenic potential." This applies not only to group A streptococci, since it has also been found that some group C (11) and group G (17) streptococci isolated in association with nephritis are also producers of bacteriocin-like substances. Although a direct role for bacteriocins in nephritis or rheumatic fever seems unlikely, the results of the present study do suggest that these substances could prove to be interesting markers of streptococci capable of participating in these enigmatic diseases.

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