APPLIED MICROBIOLOGY, Nov. 1974, p. 851-855
Vol. 28, No. 5

Printed in U.S.A.

Fluorescent Antibody Study of the Gram-Positive Anaerobic Cocci

RICHARD K. PORSCHEN AND EARLE H. SPAULDING

Department of Microbiology and Immunology, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140

Received for publication 17 July 1974

Fluorescent antibody conjugates were prepared from five species of anaerobic cocci commonly isolated from human infections. When tested with homologous and heterologous cells these conjugates were found to be highly specific. There was no evidence of a common genus antigen. *Peptococcus magnus* conjugates detected a species-specific antigen; cross-reactions with *Peptostreptococcus anaerobius* were readily eliminated by absorption. The conjugates from *Peptococcus asaccharolyticus, Peptococcus prevotii, Peptostreptococcus, anaerobius*, and *Peptostreptococcus intermedius* displayed a high degree of strain specificity. Occasional cross-reactions were detected with homologous strains, suggesting the presence of common antigens, but no attempt was made to determine the number of different serotypes in these species.

Anaerobic gram-positive cocci make up the family *Peptococcaceae* which includes the genera *Peptococcus, Peptostreptococcus, Ruminococcus*, and *Coprococcus* (11, 17). The commonly isolated anaerobic cocci from human clinical material are *Peptococcus* and *Peptostreptococcus* species. Unfortunately, differentiation of these two genera, and speciation within them, is difficult to achieve because as a group the members are highly variable in morphologic, cultural, and metabolic properties, and they are relatively inert biochemically (3, 4, 10, 14, 18, 21). Application of gas-liquid chromatography is helpful, but the majority of presently recognized species fail to show distinctly different metabolic end-product patterns (10, 14).

The identification of anaerobic cocci in most clinical microbiology laboratories is limited to colonial morphology on anaerobic blood agar plates, cellular morphology, the presence or absence of a pungent odor, and an anaerobic or microaerophilic growth requirement (5, 13, 19). Nevertheless, practical species identification of anaerobic gram-positive cocci from human sources would be desirable. Certain species are common constituents of the normal human microflora (10). They are also frequently isolated from clinical specimens. Twenty-six percent of the anaerobic isolates in a Mayo Clinic report (14), and 26% in our own laboratory (19), were identifiable species of *Peptococcus* and *Peptostreptococcus*. Their role in the etiology of human infections is unclear, but they have been so implicated in numerous reports (1, 2, 7, 12, 13, 23). It is unlikely that our understanding of their pathogenic significance will be advanced appreciably until a large number of well-characterized strains from both normal and infected sites have been appropriately examined.

We are aware of very few attempts to classify anaerobic cocci by immunological methods. In 1940, Stone (20) tried to group 26 human strains of anaerobic streptococci from obstetrical sources by bacterial agglutination and precipitin tests, but was unable to do so satisfactorily. Since then considerable attention has been directed to the classification of anaerobic gram-positive cocci by cultural and biochemical means (3, 6, 7, 10, 11, 15, 17, 22), but immunological methods have been neglected. Neither of two chapters in recent texts (13, 18) mentions these procedures. We agree with Hare (7) that "the most satisfactory method for classification of bacteria is by serology," and consequently decided to look at this approach, with emphasis upon fluorescent antibody techniques. It was our hope that the results would suggest a promising approach to the differentiation of *Peptococcus* from *Peptostreptococcus* and to the rapid identification of those species most often recovered from clinical sources.

MATERIALS AND METHODS

Test strains. All bacterial strains used in the preparation and evaluation of our fluorescent anti-

body conjugates were obtained from clinical specimens received at Temple University Health Sciences Center. They were speciated by the Anaerobe Laboratory, using the methods of the Virginia Polytechnic Institute Anaerobe Laboratory (10) which included Gram-stain reaction, colonial morphology, biochemical reactions, and gas-liquid chromatography patterns. The cocci were the more commonly isolated anaerobic and microaerophilic species *P. magnus*, *P. asaccharolyticus*, *P. prevotii*, *P. (Streptococcus) anaerobius*, and *P. intermedius*. In addition, a panel of 10 organisms (*Veillonella parvula*, *V. alcalescens*, *Bacteroides fragilis*, *Eubacterium* sp., *Bifidobacterium* sp., *Staphylococcus aureus*, *S. epidermidis*, and *Streptococcus* groups A, B, and D) were used for evaluation of the conjugates.

Immunization. Organisms to be used as antigen were cultured for 72 h on brain heart infusion agar (BBL), supplemented with 5% defibrinated sheep blood, 0.5% yeast extract, and 0.5 μg of menadione per ml. The cells were harvested with phosphate-buffered saline, pH 7.2, washed three times, and resuspended in the same solution. At least two New Zealand white rabbits (3 kg) were immunized with each antigen. Two methods of immunization were used. By the first method, the rabbits received three successive daily doses of 0.5 ml of a bacterial suspension (MacFarland no. 3 standard) intravenously, followed by a 7-day rest period, then a second and third course of injections using 1.0-ml volumes and the same rest periods. The second method used an emulsion consisting of equal volumes of a bacterial suspension (MacFarland no. 5 standard) and Freund incomplete adjuvant (Difco). One milliliter of the emulsion was injected subcutaneously in multiple sites in the nuchal area and hind foot pads, and the same dose was repeated 14 days later. A third dose of 1.0 ml of a bacterial suspension (MacFarland no. 3 standard), without adjuvant, was injected intravenously after another 14-day interval. Each rabbit was bled 7 days after its last injection. A further course of intravenous injections was given if the indirect fluorescent antibody titer (IFA) was not at least 1:640, or if additional antiserum was needed several weeks after the last bleeding. Antisera was divided into 1.0- and 5.0-ml portions and stored at 0 to 5 C. Antisera collected from the same rabbits at different dates and antisera prepared in different rabbits with the same strains were pooled for the preparation of fluorescent antibody (FA) conjugates.

Titration of antisera. Bacterial agglutination tests were performed with organisms grown on supplemented brain heart infusion. They were harvested, washed twice, and resuspended in buffered saline. Rabbit sera were titrated first by bacterial agglutination tests, using 2-fold serial dilutions and a standardized suspension of cells (one-half of MacFarland no. 1 standard). These tests were read after incubation at 37 C for 2 h and again after overnight incubation at 8 C. Autoagglutination was a problem, as previously reported by Stone (20), and recommended methods for eliminating it (16) were not successful. Therefore, the use of agglutination tests was discontinued early in this study.

Titrations of sera before and after immunization were routinely performed by the IFA technique. Suspensions of organisms (prepared as above) were spotted on slides using a calibrated bacterial loop (0.01 ml), and were air-dried at room temperature. The slides were fixed with 95% ethanol for a minimum of 1 min and the preparation was dried as before. These prepared slides were stored at −20 C or used immediately for IFA staining. Before use, rabbit sera were inactivated at 56 C in a water bath for 30 min. The smears were overlaid with 0.03 ml of rabbit antiserum or normal rabbit control serum, and incubated in a moist chamber at 37 C for 30 min. The slides were soaked twice in baths of 0.01 M phosphate buffer, pH 7.6, for 5 min each time, rinsed in distilled water, and allowed to air-dry.

Fluorescein-conjugated goat anti-rabbit globulin (Burroughs Wellcome Co., and BBL) was used in the IFA procedure and stored at 0 to 5 C in 0.5-ml portions. The working dilutions of goat globulin conjugate were determined by mixing 2-fold dilutions with the highest dilution of antiserum giving a 4+ staining reaction with autologous cells. Prepared smears (bacteria reacted with rabbit antibody) were overlaid with 0.03 ml of the conjugate, then incubated, soaked, and dried as described above. A drop of glycerine buffered with 0.01 M phosphate, pH 9.0, was placed over the preparation, and a cover slip was added. The IFA titers were determined as the highest dilution of antisera producing 4+ fluorescence. The immunofluorescence of homologous and heterologous cell preparation was determined with rabbit antisera diluted 1:500. Intensity of fluorescence was rated 0 to 4+, a reaction of 2+ or greater being considered positive.

Preparation of FA conjugates. Pre- and post-immunization globulins were obtained by three precipitations of sera with 70% saturated ammonium sulfate (8, 9). The precipitates were dissolved in distilled water and dialyzed at 4 C against frequent changes of 0.85% NaCl solution, pH 8, until free of ammonium sulfate. The protein concentration, determined by the biuret method, was adjusted to approximately 15 mg/ml with phosphate-buffered saline, pH 7.2. The globulins were conjugated with fluorescein isothiocyanate (FITC; BBL) by the direct method, using labeling conditions of 25.0 μg of FITC per mg of protein for 2.5 h. Uncombined fluorescein was removed by dialysis against buffered saline, pH 7.6. The labeled globulins were merthiolated (1:10,000; Lilly) and stored at 0 to 5 C.

Direct FA staining. The procedure was essentially the same as that described for IFA staining. The difference was that the spots of antigen were covered with 0.03 ml of conjugated rabbit antisera or normal sera. The antiserum titers were the highest dilutions providing 4+ fluorescence with the autologous strain. The working dilution for testing different cells was one doubling dilution lower than the highest dilution giving maximal fluorescence with the autologous strain. Positive reactions were categorized by a 2+ or greater fluorescence.

Stained preparations were examined with a Zeiss

fluorescence microscope equipped with an Osram HBO 200 mercury lamp, oil immersion dark-field condenser, a BG 12 exciter filter, and a 53/9/44 barrier filter.

Absorption of antiserum. Absorption of cross-reacting antibody was performed by mixing equal volumes of conjugated antiserum and packed cells from a 48-h supplemented blood agar culture of the heterologous, cross-reacting strain. The mixture was incubated at 37 C for 2 h. This procedure was repeated when necessary.

RESULTS

Two methods of immunization were used: (i) intravenous injections of bacterial suspensions, and (ii) subcutaneous injections of bacterial cells plus Freund incomplete adjuvant, followed by bacterial suspension without adjuvant administered intravenously. The antibody titers with the two methods were about the same; therefore, antisera data were combined. There was too little cross-reactivity to judge whether there was a significant difference in the specificity between the antisera elicited by the two methods.

Antibody titers as determined by the indirect fluorescence staining technique for the 11 autologous strains are shown in Table 1. They fall within the narrow range of 1:640 and 1:2,560. Thus there was no indication that one species or one strain within a species was any more immunogenic than the others. No preimmunization serum had a titer greater than 1:20. There were no cross-reactions with heterologous species with antiserum dilutions 1:500 or greater.

Antisera produced with the same strains, and having IFA titers of 1:640 or greater, were pooled and conjugated with FITC. Table 2 shows the direct FA staining titers of the conjugates with autologous cells. These ranged from 1:4 to 1:32. There were no positive reactions with conjugated rabbit sera from unimmunized rabbits.

The specificity of conjugated antisera was evaluated by reactions with a panel of 49 homologous and heterologous strains of anaerobic gram-positive cocci. A high degree of specificity was observed with these conjugates. There was little evidence of cross-reactivity with organisms of heterologous species with the exception of *P. anaerobius* strains cross-reacting with both *P. magnus* conjugates. A single absorption reduced this reactivity to a negative reading. Additionally, the conjugates did not react with other heterologous organisms including anaerobic gram-negative cocci and bacilli, as well as aerobic streptococci and staphylococci.

TABLE 1. *Indirect fluorescence staining titers of antisera with autologous cells*

Antisera[a]	Reciprocal of highest dilution showing 4+ staining
Peptococcus magnus (998-1)	1,280
P. magnus (926-2)	1,280
P. asaccharolyticus (1056-9)	2,650
P. asaccharolyticus (991-1)	640
P. prevotii (937-1)	2,560
P. prevotii (881-1)	640
P. prevotii (519-2)	1,280
Peptostreptococcus anaerobius (937-6)	640
P. anaerobius (1018-4)	1,280
P. intermedius (558-5)	640
P. intermedius (1039-1)	1,280

[a] Preimmunized sera did not have titers greater than 1:20.

TABLE 2. *Staining titers of FA conjugates with autologous cells*

Conjugate	Reciprocal of highest dilution showing 4+ staining
Peptococcus magnus (998-1)	32
P. magnus (926-2)	16
P. asaccharolyticus (1056-9)	32
P. assacharolyticus (991-1)	4
P. prevotii (937-1)	32
P. prevotii (881-1)	4
P. prevotii (519-2)	8
Peptostreptococcus anaerobius (937-6)	8
P. anaerobius (1018-4)	16
P. intermedius (558-5)	4
P. intermedius (1039-1)	16

Table 3 contains the results with homologous strains. *P. magnus* conjugates (998-1 and 926-2) were unique in that marked species-specific reactivity was observed. The intensities of these reactions were mostly 3+ and 4+. There were no positive reactions with cells of other *Peptococcus* species. *P. asaccharolyticus* conjugates (1056-9 and 991-1), on the other hand, reacted with only two and one homologous strains, respectively, but not with each other. Neither of the two strains that reacted with 1056-9 reacted with 991-1. The conjugates from three strains of *P. prevotii* (937-1, 881-1, and 519-2) were completely strain specific; they did not react with any of the 12 homologous strains tested. *P. anaerobius* 937-6 and 1018-4, and *P. intermedius* 558-1 and 1039-1, conjugates were also highly strain specific. *P. anaerobius* 937-6

TABLE 3. *Staining reactions of FA conjugates with homologous strains*

Conjugate	Homologous strains[a]	
	Reactions	
	Positive	Negative
Peptococcus magnus (998-1)	9	1
P. magnus (926-2)	10	0
P. asaccharolyticus (1056-9)	2	11
P. asaccharolyticus (991-1)	1	12
P. prevotii (937-1)	0	12
P. prevotii (881-1)	0	12
P. prevotii (519-2)	0	12
Peptostreptococcus anaerobius (937-6)	2	7
P. anaerobius (1018-4)	0	9
P. intermedius (558-5)	0	5
P. intermedius (1039-1)	1	4

[a] Evaluation of the conjugates included at least duplicate trials with homologous cells.

and *P. intermedius* 1039-1 conjugates reacted with two and one homologous strains, respectively.

DISCUSSION

To our knowledge there has been no report of anaerobic gram-positive cocci speciated by Virginia Polytechnic Institute methods (10) having been examined by immunological methods. This exploratory study was designed to determine by fluorescence microscopy the presence or absence of major genus and species-specific surface antigens in certain species. Our immediate goal was to obtain data suggesting a useful application of this technique in the clinical microbiology laboratory, a clear need in view of the difficulty with which members of this group are at present identified. In addition, we hoped that the results would point the way for subsequent work.

The five species selected for study were chosen because they are the ones most often recovered in our laboratory from infected sites in human beings. Over a 3-year period, *P. prevotii* accounted for 6%, *P. anaerobius* for 4.8%, *P. intermedius* for 4.1%, *P. asaccharolyticus* for 3.9%, and *P. magnus* for 3.6% of all anaerobic isolates (unpublished data). We decided that 2 or 3 carefully selected strains of each species would suffice for the limited purposes of this study. Whether or not the results with these few strains accurately reflect the overall presence or absence of major genus and species-specific antigens among these five species is a question that can be answered only by testing many more strains than the 49 used here.

We failed to obtain any indication of a common *Peptococcus* or *Peptostreptococcus* antigen. Furthermore, tests with three different *P. prevotii* antisera and a panel of 12 strains were uniformly negative, suggesting that the members of this species may to a large degree be strain specific. The cross-reactions observed with one strain each of *P. anaerobius* and *P. intermedius*, and both strains of *P. asaccharolyticus* demonstrated that shared surface antigens do exist within these species, but how many there are must await further study.

The results with *P. magnus*, however, were different. With a single exception, all 10 strains in the test panel reacted strongly with both homologous antisera. It is reasonable to expect, therefore, that a *P. magnus* 926-2 antiserum will detect at least a large majority of strains belonging to this species. As noted earlier, the cross-reactivity with *P. anaerobius* strains was readily removable. Because neither of the *P. magnus* antisera working dilution cross-reacted with any of the other 25 test strains of *Peptococcus*, it appears that this organism contains a major species-specific surface antigen detectable by fluorescence microscopy. The clear-cut nature of these results suggest that fluorescence technique may be a practical means for detecting *P. magnus* in the clinical laboratory.

The existence of several, and perhaps numerous, serotypes within the other four species can be expected. For example, the two *P. asaccharolyticus* strains used as immunogens did not react with each other, and the test strain reacting with 1056-9 antiserum did not react with the 991-1 antiserum. Additionally, the two test strains reacting with 991-1 did not react with 1056-9 antiserum. The feasibility of developing useful polyvalent antisera for the specific identification of these species can be decided only after considerably more work.

The investigation of the immunological properties of well-characterized strains of anaerobic gram-positive cocci should be pursued. At the present time they constitute a problem in taxonomy. They show considerable variation in morphology. As a group they are generally inert biochemically; in addition, the more active species are quite inconsistent in their cultural and biochemical properties. Gas-liquid chromatography is a helpful procedure but is not definitive, and the equipment for it is as yet unavailable in most clinical microbiology laboratories.

The role of anaerobic gram-positive cocci as causative agents in human infections is poorly understood. For example, studies in this laboratory with a rabbit experimental infection model suggest that *P. asaccharolyticus*, *P. magnus*, *P. anaerobius*, and *V. parvula* fail to produce persisting subcutaneous lesions, whereas *V. alcalescens* does so quite consistently (M. J. Widomski, E. H. Spaulding, and R. M. Swenson, Abstr. Ann. Meet. Amer. Soc. Microbiol., 1974, M343, p. 123). Significant advances in our knowledge of their pathogenicity will be greatly accelerated, therefore, if practical serological tests can be developed for dependable identification of these five species.

ACKNOWLEDGMENTS

The valuable assistance of Sandra Sonntag, Matthew Widomski, and the staff of the Anaerobe Laboratory is acknowledged with appreciation.

This study was supported in part by Medical and Public Health Service Microbiology Postdoctoral Training grant 5 TO1 AI00233.

LITERATURE CITED

1. Anderson, C. B., J. J. Marr, and B. M. Jaffe. 1972. Anaerobic streptococcal infections simulating gas gangrene. Arch. Surg. **104**:186-189.
2. Bornstein, D. L., A. N. Weinberg, M. N. Schwartz, and L. J. Kunz. 1964. Anaerobic infections—review of current literature. Medicine **43**:207-232.
3. Breed, R. E., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology, 7th ed. Williams and Wilkins Co., Baltimore.
4. Dowell, V. R., Jr., and T. M. Hankins. 1974. Laboratory methods in anaerobic bacteriology: Center for Disease Control laboratory manual no. 74-8272. Center for Disease Control, Atlanta, Ga.
5. Ellner, P. D., P. A. Granato, and C. B. May. 1973. Recovery and identification of anaerobes: a system suitable for the routine clinical laboratory. Appl. Microbiol. **26**:904-913.
6. Foubert, E. L., and H. C. Douglas. 1948. Studies on the anaerobic micrococci. I. Taxonomic considerations. J. Bacteriol. **56**:25-34.
7. Hare, R. 1967. The anaerobic cocci, p. 284-317. *In* A. P. Waterson (ed.), Recent advances in medical microbiology. Little, Brown and Co., Boston, Mass.
8. Hebert, G. A., P. L. Pelham, and B. Pittman. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. Appl. Microbiol. **25**:26-36.
9. Hebert, G. A., B. Pittman, R. M. McKinney, and W. B. Cherry. 1972. The preparation and physiochemical characterization of fluorescent antibody reagents. Center for Disease Control, Atlanta, Ga.
10. Holdeman, L. V., and W. E. C. Moore. 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg, Va.
11. Holdeman, L. V., and W. E. C. Moore. 1974. New genus, coprococcus, twelve new species, and emended descriptions of four previously described species of bacteria from human feces. Int. J. Syst. Bacteriol. **24**:260-277.
12. Lambe, D. W., D. H. Vroon, and C. W. Rietz. 1973. Infections due to anaerobic cocci, p. 585-599. *In* A. Balows et al. (ed.), Anaerobic bacteria: role in disease. Charles C Thomas, Springield, Ill.
13. Martin, W. J. 1974. Anaerobic cocci, p. 381-387. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
14. Pien, F. D., R. L. Thompson, and W. J. Martin. 1972. Clinical and bacteriologic studies of anaerobic gram-positive cocci. Mayo Clin. Proc. **47**:251-257.
15. Prevot, A. R. Manuel de classification et de determination des bacteries anaerobies. Masson, Paris.
16. Rogosa, M. 1965. The genus *Veillonella*. IV. Serological groupings, and genus and species emendations. J. Bacteriol. **90**:704-709.
17. Rogosa, M. 1971. *Peptococcaceae*, a new family to include the gram-positive anaerobic cocci of the genera *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus*. Int. J. Syst. Bacteriol. **21**:234-237.
18. Smith, L. DS., and L. V. Holdeman. 1968. The anaerobic cocci, p. 81-95. *In* The pathogenic anaerobic bacteria. Charles C Thomas, Springfield, Ill.
19. Spaulding, E. H., V. Vargo, T. C. Michaelson, M. Korzeniowski, and R. M. Swenson. 1974. Anaerobic bacteria: culture and identification, p. 87-94. *In* J. E. Prier and H. Friedman (ed.), Opportunistic pathogens. University Park Press, Baltimore.
20. Stone, M. L. 1940. Studies on the anaerobic streptococcus. I. Certain biochemical and immunological properties of anaerobic streptococci. J. Bacteriol. **39**:559-582.
21. Sutter, V. L., H. R. Atteberg, J. E. Rosenblatt, K. S. Bricknell, and S. M. Finegold. 1972. Anaerobic bacteriology manual. University of California at Los Angeles, Los Angeles.
22. Thomas, C. G. A., and R. Hare. 1954. The classification of anaerobic cocci and their isolation in normal human beings and pathologic processes. J. Clin. Pathol. **7**:300-304.
23. Weinberg, A. N. 1973. Infections due to anaerobic cocci, p. 257-265. *In* A. Balows et al. (ed.), Anaerobic bacteria: role in disease. Charles C Thomas, Springfield, Ill.