JOURNAL OF BACTERIOLOGY, Dec., 1966
Vol. 92, No. 6

Differentiation of Cariogenic Streptococci by Fluorescent Antibody[1]

JAMES M. JABLON AND DORAN D. ZINNER
Department of Microbiology, University of Miami, School of Medicine, Miami, Florida

Received for publication 24 August 1966

ABSTRACT

JABLON, J. M. (University of Miami, Miami, Fla.), AND D. D. ZINNER. Differentiation of cariogenic streptococci by fluorescent antibody. J. Bacteriol. 92:1590–1596. 1966.—Eight strains of streptococci were isolated from human carious lesions by the fluorescent-antibody (FA) technique. Seven of these strains produced experimental caries in hamsters or rats maintained on a high sucrose diet. The eighth strain was noncariogenic in animals but possessed some antigenic components in common with the cariogenic strains. On the basis of antigen-antibody reactions by microprecipitin and agar-gel diffusion patterns, the strains were divided into four groups; these groups differed with regard to their cariogenic activity in hamsters. Fluorescein-conjugated antisera, prepared against the human strains, showed some cross-reactions which interfered with the efficacy of the FA technique in differentiating between the related streptococcal groups. To eliminate these cross-reactions, a small amount of related-strain antisera was added to the fluorescein-conjugated antisera to the cariogenic strains. This technique is effective in blocking cross-reactions and should be tried wherever cross-reactions are encountered in the FA technique.

The recent work of Orland et al. (13), Fitzgerald et al. (4), and Fitzgerald and Keyes (5), in experimental dental caries in rats and hamsters, has indicated the possible role of certain strains of streptococci in the etiology of this disease. However, the abundant presence of various bacterial species in human oral flora made the identification of similar streptococcal strains difficult in human caries.

The fluorescent-antibody (FA) technique (2, 3) has been used in recent years as a diagnostic procedure for the identification of bacteria and viruses (1). As such, it has been used for the rapid identification of group A streptococci in throat cultures (12). In this procedure, the FA technique had limitations, because cross-reactions were sometimes encountered with groups C and G streptococci (12, 16), organisms which are commonly present in the human oral flora but are lacking the pathogenic potential of group A organisms. These organisms contain antigenic components closely related to group A. The cross-reactions in the FA technique were eliminated by the adsorption of the fluorescein-conjugated group A antiserum with group C cells, followed by the addition of a small amount of unconjugated group C antiserum to the fluorescein-conjugated group A antiserum (15).

In the present study, the FA technique was adapted to use for the rapid identification of streptococci in the human oral flora having cariogenic potential under appropriate experimental conditions. Through use of the animal strains of cariogenic streptococci, antisera were prepared and conjugated with fluorescein isothiocyanate; these antisera facilitated the identification of similar strains in human carious lesions (18). Eight human strains were isolated (J. M. Jablon, D. D. Zinner, and A. P. Aran, Bacteriol. Proc., p. 59, 1966; D. D. Zinner et al., Intern. Assoc. Dental Res. Abstr., p. 52, 1966); seven of these produced experimental caries in hamsters or rats, at different rates (D. D. Zinner et al., Arch. Oral Biol., *in press*), whereas the eighth was antigenically related but was noncariogenic. Antisera were prepared against the human strains and used in the FA technique to determine the prevalence of cariogenic streptococci in the human oral flora of individuals with and without active caries. During the study, cross-reactions with antigenically related strepto-

[1] Presented in part at the 66th Annual Meeting of the American Society for Microbiology, Los Angeles, Calif., 1–5 May 1966.

cocci were encountered. Attempts to eliminate the cross-reactions by adsorbing the antisera with the related strains were unsuccessful; the resultant antisera were too weak for use. In the present report, the cross-reactions were eliminated by the addition of a small amount of antigenically related streptococcal antisera to the fluorescein-conjugated streptococcal antisera. The technique is similar to that used by Redys, Ross, and Borman (16) for group A streptococcal antiserum, except that preliminary adsorption with common antigenic streptococcal strains was not necessary.

MATERIALS AND METHODS

Cultures. Animal strains of cariogenic streptococci, HS-1 (a hamster strain) and FA-1 (a rat strain), were obtained from Robert Fitzgerald, National Institute of Dental Research, Bethesda, Md. Human strains, AHT, BHT, CHT, DHT, EHT, FHT, HHT, and IHT, were isolated from human oral flora or carious lesions. Of these, CHT was a noncariogenic strain which contained some antigenic components in common with the cariogenic streptococci. For simplicity, all the human strains will be labeled by the first letter only (A, B, C, etc.), since the last letters, HT, signify that their source is human teeth.

Media. All cultures were grown in Todd-Hewitt Broth (Difco) supplemented with 0.5% lactalbumin hydrolysate. For a solid medium, Mitis Salivarius Agar (Difco) supplemented with 1.0 ml of 1% Chapman Tellurite Solution (Difco) per liter was used. This medium inhibited gram-negative organisms and served as an excellent medium for the isolation of typical colonies.

Antisera. Originally, heat-killed vaccines (60 C for 30 min) were prepared from the hamster and rat strains of cariogenic streptococci, and rabbits were immunized by intravenous injections on alternate days, according to a previously described schedule (8). Later, when the human strains were isolated, antisera were also prepared against them.

Fluorescein conjugation of antisera and technique of staining. The technique of antiserum conjugation with fluorescein isothiocyanate and of staining of streptococcal cultures was that outlined by Moody et al. (12). The fluorescein-conjugated antisera were generally used in a dilution of 1:50, with phosphate-buffered saline (*p*H 7.2) as the diluent. These conjugated antisera were prepared against strain HS-1, strain FA-1, and human strains A, B, C, and H; the remaining antisera were not conjugated, because, as will be noted later, the A and B strains are representative of the other strains.

Immunological tests. All the prepared antisera were tested by microprecipitin (17) and Ouchterlony techniques (14), using Lancefield (11) acid extracts of the various streptococcal strains. The microprecipitin tests indicated both specific reactions and cross-reactions, and the Ouchterlony technique indicated the relationship between the reacting antigen-antibody components. This technique was run in the usual manner using 3 × 1 inch (7.6 × 2.5 cm) slides. The slides were placed in an LKB immunoelectrophoresis slide frame (LKB Instruments, Inc., Washington, D.C.) and coated with 1% agar (Difco). After solidification, a trough 2 mm wide and 65 mm long was cut in the agar through the middle and along the length of each slide, with an LKB trough cutter, and wells, 2 mm in diameter, were cut above and below the trough. With this modification in the design of the Ouchterlony technique, 12 extracts could readily be accommodated on the same slide to show antigen-antibody relationships. The antiserum was placed in the trough and the streptococcal extracts were placed above and below the troughs. The frames containing the slides were placed in holders, and diffusion was allowed to proceed for 48 hr at room temperature in a moist atmosphere. The excess, nonreactive serum was then washed from the slides by placing them in several changes of 1% saline in a polyethylene container for 2 days. To facilitate the removal of the excess serum, a magnetic stirrer was used. The holder containing the slides was then immersed in distilled water for 1 hr; the slides were then covered with strips of filter paper moistened with distilled water, left to dry overnight in a 37 C incubator, and stained with 0.1% amido black by the technique of Hirschfeld (7). The dark antigen-antibody bands were then photographed.

Isolation of streptococci from human teeth. In a previous study (18), material was taken from active human carious lesions with a spoon excavator, was placed in a tube containing 5 ml of Todd-Hewitt Broth (Difco) supplemented with lactalbumin hydrolysate, and was incubated overnight at 37 C. The isolation of typical colonies, under these conditions, was often difficult, because the cariogenic streptococci were frequently overwhelmed by the more rapid growth of other oral microorganisms. It was found that incubation of the mixed oral flora for 4 to 5 hr was sufficient to give the cariogenic streptococci a good start in growth without being overgrown by other flora. Typical chains can readily be distinguished at this time by the FA technique. At the same time, a loopful of material was streaked on Mitis Salivarius Agar, and in 48 to 72 hr typical A and B colonies, if present, could be readily seen. These colonies have been previously described (18). The A colonies form a gelatinous substance, which is probably an extracellular carbohydrate (6), whereas the B and C colonies do not. The H colonies, which have not been previously described, have the thick and gummy appearance of *Streptococcus salivarius*. They grow much more rapidly than either the A, B, or C colonies, and appear after overnight incubation. The D, E, and F colonies resemble the A strain and the HS-1 strain, whereas the I colonies resemble the B strain and the FA-1 strain.

Testing for cariogenic activity. The human strains were tested for cariogenic activity in 19-day-old hamsters (weighing about 30 g) on a high sucrose diet. The animals were orally infected and were then examined periodically for caries formation. The teeth were examined by stereomicroscopy after the animals had been anesthetized by an intraperitoneal injection

of 0.1 ml per mg of body weight of pentobarbital sodium (50 mg/ml) diluted 1:10 with water. When different degrees of caries formation were revealed by stereomicroscopy, the animals were sacrificed and decapitated. The jaws were stripped, and the dental arches were examined. The extent of the caries involvement was estimated by the caries-scoring technique of Keyes (9), in which the number of teeth surfaces affected and the depth of the lesions were evaluated. In the present work, the caries involvement was subdivided into: initial caries, where lesions were superficial and few in number; moderate caries, where many sections of teeth surfaces and deeper lesions were apparent; and total caries, in which there was almost complete destruction of teeth.

RESULTS

Microprecipitin tests. Acid extracts (11) of the cultures of the animal strains (HS-1 and FA-1) and of the eight human strains (cariogenic strains A, B, D, E, F, H, and I and the antigenically related noncariogenic strain C) of streptococci were used in the microprecipitin technique (17) with the various antisera.

The results (Table 1) show that the hamster (HS-1) strain and the human A, D, E, and F strains are closely related. The cross-reactivity of the C and H extracts with the HS-1 and A antisera, which did not occur with the D, E, and F antisera, was apparent only as a slight precipitate after overnight refrigeration. However, the major reactions would tend to place the HS-1, A, D, E, and F strains in a single group. In the same manner, the rat (FA-1) strain and the B and I strains may be placed in a second group. Strain C and strain H belong to individual separate categories, although they exhibit considerable cross-reactivity with each other and with the other strains.

Agar-gel (Ouchterlony) tests. The tentative grouping of the streptococcal strains, based on the presence of common antigenic components, is best brought out by the Ouchterlony technique (14). The results are illustrated in Fig. 1. The antisera, indicated by capital letters, were placed in the troughs, and the streptococcal acid extracts (11), shown in lower-case letters, were placed above and below the troughs. With the A antiserum (AHT), there was a reactive identity between the extracts of strains HS-1, A, D, E, and F (designated in the figure as *hs*, *a*, *d*, etc.), thus placing them in one group. The reactions of the FA, B, and I strains with the BHT serum places them in a second group. With the C (CHT) and H (HHT) antisera, extracts of only the C and H strains (designated as *c* and *h*) showed reactive bands which were different from each other. This placed these two strains in separate groups.

Cariogenic activity. The relative ability of the streptococcal groups to induce caries in hamsters maintained on a high sucrose diet was investigated. The animals were orally infected, and, from time to time, the teeth were examined by stereomicroscopy. When different degrees of caries were noted, the animals were sacrificed. The results of the comparative rates of the caries activity of the different groups are in Table 2.

The above results indicate that the separation of the strains into groups on the basis of antigen-antibody reactions can be extended to include caries activity. On this basis, group 1 was the most active, group 3 was the least active, and group 4 was completely without caries activity.

FA reactions. Fluorescein-conjugated antisera to the animal strains and to human strains A, B, C, and H, as representatives of the tentative streptococcal categories, were used to check

TABLE 1. *Microprecipitin reactions of cariogenic and related oral streptococci*

Group	Antiserum	Bacterial extracts									
		HS-1	A	B	C	D	E	F	H	I	FA-1
I	HS-1	4+	3+	—	1+	3+	3+	3+	1+	—	—
	A	3+	3+	—	1+	3+	3+	3+	1+	—	—
	D	4+	4+	—	—	4+	3+	2+	—	—	—
	E	3+	3+	—	—	3+	3+	3+	—	—	—
	F	3+	3+	—	—	3+	3+	3+	—	—	—
II	B	—	—	4+	1+	—	—	—	2+	4+	4+
	I	—	—	3+	—	—	—	—	2+	3+	3+
	FA-1	—	—	3+	2+	—	—	—	1+	3+	4+
III	H	—	—	—	3+	—	—	—	4+	—	—
IV	C	—	—	1+	4+	—	—	—	2+	—	2+

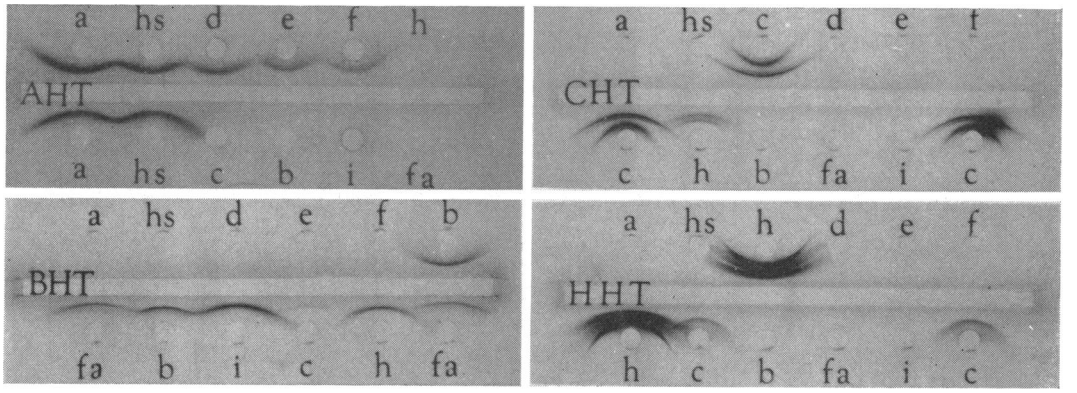

FIG. 1. *Agar-gel diffusion reactions. Antisera in the troughs in capital letters; streptococcal extracts of human strains and hamster (hs) and rat (fa) strains are indicated in lower-case letters.*

TABLE 2. *Comparative cariogenic activity of human streptococcal strains in hamsters*

Streptococcal strain	Group	Elapsed time (days) after initial oral infection		
		Initial caries[a]	Moderate caries[b]	Total caries[c]
HS-1	1	26–30	40–55	75–90
AHT				
DHT				
EHT				
FHT				
FA-1[d]	2	65–70	100–110	150–170
BHT				
HHT	3	90–100	130–140	180–200
CHT	4	NC[e]	NC	NC

[a] Initial caries = lesions few and superficial.
[b] Moderate caries = many lesions, some deep.
[c] Total caries = almost total destruction of the teeth.
[d] FA-1 culture produces caries in gnotobiotic rats; not tested in hamsters.
[e] No caries in more than 200 days.

possible cross-fluorescence reactions to the various strains (Table 3).

The fluorescence of strains HS-1, A, D, E, and F with the HS-1 and A fluorescein-conjugated antisera, and that of strains FA-1, B, and I with the FA-1 and B antisera, corroborates the close relationship of the strains indicated by the microprecipitin (17) and Ouchterlony (14) tests. The H strain also showed a relationship because of cross-reactivity of the fluorescence reactions with all the antisera. The C strains showed an aberrant reaction in which the fluorescence was limited to the outer periphery of the organism when stained with the A, HS-1, B, or FA-1 fluorescein-conjugated antisera, indicating at least a partial antigenic relationship. This aberrant fluorescence reaction has been reported previously (18). The significance of this reaction is not apparent at present, but it may indicate a lack of homogeneity in the cell wall of this strain. An example of the aberrant, one-sided fluorescence is presented in Fig. 2, in which a mixture of A, B, and C cultures was stained with fluorescein-conjugated A

TABLE 3. *Reactions of cariogenic and related streptococci with fluorescein-tagged antisera*

Fluorescent antiserum	Streptococcal strains									
	HS-1	FA-1	A	B	C	D	E	F	H	I
HS-1	4+	—	4+	—	A[a]	4+	4+	4+	3+	—
FA-1	—	4+	—	4+	A	—	—	—	3+	4+
A	4+	—	4+	—	A	4+	4+	4+	3+	—
B	—	4+	—	4+	A	—	—	—	4+	4+
C	—	—	—	—	4+	—	—	—	3+	—
H	—	—	—	—	A	—	—	—	4+	—

[a] Signifies an aberrant reaction. The fluorescence is limited to one segment of the outer periphery of the organism only.

antiserum. The A strain showed complete fluorescence, the B strain did not fluoresce with this antiserum, and the C strain showed the one-sided aberrant fluorescence. The same aberrant reaction took place with the B fluorescein-tagged antiserum.

Elimination of cross-reactions with fluorescein-tagged antisera. An attempt was made to elimi-

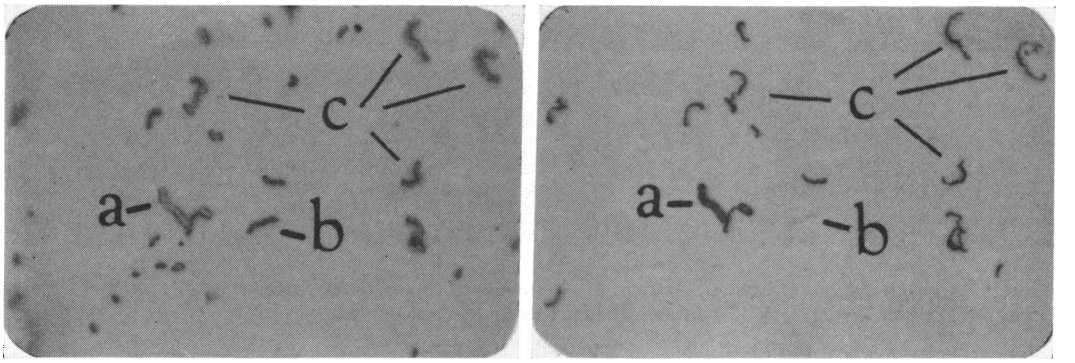

FIG. 2. *Specific and aberrant fluorescence reactions. Mixture of a, b, and c streptococcal strains stained with fluorescein-conjugated A antiserum. Left side is in dark-field; right side is same field under fluorescence. Note that the a strain shows complete fluorescence, the b strain does not fluoresce, and the c strain shows aberrant fluorescence.*

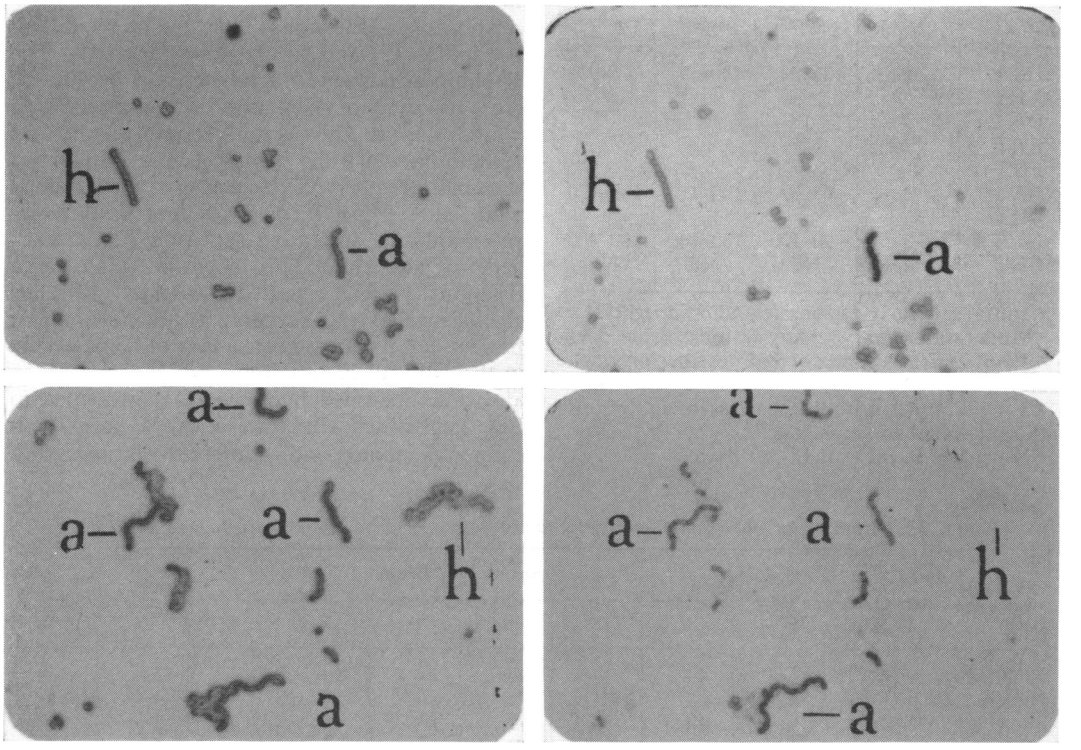

FIG. 3. *Blocking of h strain cross-fluorescence by the addition of related-strain antisera to fluorescein-conjugated A antiserum. Upper section: cross-reaction when A antiserum alone is used; left section in dark-field, right section by fluorescence. Note that a and h strains both fluoresce, a with greater intensity. Lower section: C and H antisera are added to A fluorescein-conjugated antiserum. Left section in dark-field; right section is same area under fluorescence. Note that the h strain fluorescence is blocked while the fluorescence of the a strain is unaffected.*

nate the cross-reactions by adsorbing the antisera with C and H strains prior to conjugation with fluorescein isothiocyanate. This attempt was unsuccessful; the final adsorbed antisera were too weak for use. A technique similar to that employed by Redys, Parzick, and Borman (15) in inhibiting common antigen fluorescence in the identification of group A streptococci was then tried. In that technique, the fluorescein-conjugated group A antiserum was first adsorbed with group C streptococcal cells, and then a small amount of nonconjugated group C antiserum was added. This mixture of antisera successfully inhibited common antigen fluorescence of groups C and G streptococci as well as staphylococci. In the preceding sections, it was shown that some antigens of cariogenic streptococci are common in the noncariogenic C strain and the mildly cariogenic H strain. Therefore, a small amount of nonconjugated C and H antisera was added to the fluorescein-conjugated A and B antisera. The A and B antisera were used in a dilution of 1:50 to 1:100, and the C and H antisera were added in a final dilution of 1:50.

An example of the staining effectiveness of the mixture of fluorescein-conjugated and unconjugated antisera in inhibiting cross-reactions is presented in Fig. 3. Samples of A and H broth cultures were deliberately mixed, placed on slides, heat-fixed, and stained with the fluorescein-conjugated A antiserum. Both the A and H strains fluoresced; the A strain had greater intensity, but the H strain showed considerable cross-reactive fluorescence. The lower section of Fig. 3 shows the same culture mixture, stained with the antiserum mixture of fluorescein-conjugated A antiserum and unconjugated C and H antisera. The fluorescence of the H strain was completely inhibited, and the fluorescence intensity of the A remained unaffected. The cross-reactions with the B antiserum and the aberrant fluorescence reaction of the C strains with either the A or B antisera were similarly inhibited by the addition of the unconjugated C and H antisera.

In the present modification of the technique of Redys, Parzick, and Borman (15), it was not necessary to adsorb the fluorescein-conjugated A and B antisera with the C and H strains. The addition of the unconjugated C and H antisera was effective in blocking the cross-fluorescence reactions by these organisms. It was also found that the addition of either the C or the H antiserum alone was not as effective as adding both antisera.

DISCUSSION

In the studies on human dental caries, with the FA technique (1–3, 12), eight streptococcal strains were isolated; seven of these were cariogenic at varying rates, but the eighth was noncariogenic, although possessing antigenic components in common with some of the cariogenic strains. In the present investigation, these strains were tentatively divided into four groups based on their antigenic components as demonstrated by the microprecipitin (17) and Ouchterlony (14) techniques and by caries activity.

We encountered some cross-reactivity among the four groups of cariogenic and antigenically related noncariogenic streptococci by the FA technique. To eliminate these cross-reactions, related-strain antisera were added to the fluorescein-conjugated antisera to the cariogenic streptococci. The related-strain antisera blocked the reactions of the cross-reacting strains. This probably occurred because of the relative speed with which specific and cross-reactions occurred. In the precipitin test, specific reactions were often visible in less than 1 min; cross-reactions took place at a much slower rate and sometimes became visible only after overnight refrigeration. The FA reaction was read after a shorter time and the sensitivity was high; therefore, cross-reactions with common, secondary antigens were readily picked up. However, when the related-strain antisera were added to the specific fluorescein-conjugated antiserum, they reacted rapidly to the related, cross-reacting strains for which they were specific, forming an antigen-antibody bond which blocked the secondary reaction to the fluorescein-conjugated antiserum. Wherever cross-reactions are encountered in the FA technique, the addition of a small amount of related-strain antiserum should be considered as a means of blocking cross-reactions, instead of using the technique of adsorption which frequently results in a greatly weakened antiserum.

The FA technique is an excellent tool for the identification of cariogenic streptococci. By its use, many additional related strains may be identified and isolated. In this way, a common denominator, or denominators, may be found among the various strains which will clarify the role of the organism in the mechanism of dental caries.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research grants DE-01519 and DE-02180 from the National Institute of Dental Research.

LITERATURE CITED

1. CHERRY, W. B., M. GOLDMAN, AND T. R. CARSKI. 1960. Fluorescent antibody techniques in the diagnosis of communicable diseases. U.S. Public Health Serv. Publ. 729.
2. COONS, A. H., H. J. CREECH, AND R. N. JONES.

1941. Immunological properties of an antibody containing a fluorescent group. Proc. Soc. Exptl. Biol. Med. **47**:200–202.

3. COONS, A. H., AND M. H. KAPLAN. 1950. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exptl. Med. **91**:1–13.
4. FITZGERALD, R. J., H. V. JORDAN, AND H. R. STANLEY. 1960. Experimental caries and gingival pathologic changes in the gnotobiotic rat. J. Dental Res. **39**:923–935.
5. FITZGERALD, R. J., AND P. H. KEYES. 1960. Demonstration of the etiologic role of streptococci in experimental caries in the hamster. J. Am. Dental Assoc. **61**:9–19.
6. GIBBONS, R. J., K. S. BERMAN, P. KNOETTNER, AND B. KAPSIMALIS. 1966. Dental caries and alveolar bone loss in gnotobiotic rats infected with capsule forming streptococci of human origin. Arch. Oral Biol. **11**:549–560.
7. HIRSCHFELD, J. 1960. Immunoelectrophoresis-Procedure and application to the study of group-specific variations in sera. Sci. Tools **7**:18–25.
8. JABLON, J. M., B. BRUST, AND M. S. SASLAW. 1965. β-Hemolytic streptococci with group A and type II carbohydrate antigens. J. Bacteriol. **89**:529–534.
9. KEYES, P. H. 1944. A method of recording and scoring gross carious lesions in the molar teeth of Syrian hamsters. J. Dental Res. **23**:439–444.
10. KEYES, P. H. 1959. Dental caries in the Syrian hamster. VIII. The induction of rampant caries in albino and golden animals. J. Dental Res. **38**:525–533.
11. LANCEFIELD, R. C. 1928. The antigenic complex of streptococcus haemolytics. III. Chemical and immunological properties of species-specific substance. J. Exptl. Med. **47**:481–491.
12. MOODY, M. D., E. C. ELLIS, AND E. I. UPDYKE. 1958. Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. J. Bacteriol. **75**:553–560.
13. ORLAND, F. J., J. R. BLAYNEY, R. W. HARRISON, J. A. REYNIERS, P. C. TREXLER, R. F. ERVIN, H. A. GORDON, AND M. WAGNER. 1955. Experimental caries in germfree rats inoculated with enterococci. J. Am. Dental Assoc. **50**: 259–272.
14. OUCHTERLONY, O. 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. **26**:507–515.
15. REDYS, J. J., A. B. PARZICK, AND E. K. BORMAN. 1963. Detection of group A streptococci in throat cultures by immunofluorescence. Public Health Rept. **78**:222–226.
16. REDYS, J. J., M. R. ROSS, AND E. K. BORMAN. 1960. Inhibition of common antigen fluorescence in grouping streptococci by the antibody method. J. Bacteriol. **80**:823–829.
17. SWIFT, H. F., A. T. WILSON, AND R. C. LANCEFIELD. 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exptl. Med. **78**:127–133.
18. ZINNER, D. D., J. M. JABLON, A. P. ARAN, AND M. S. SASLAW. 1965. Experimental caries induced in animals by streptococci of human origin. Proc. Soc. Exptl. Biol. Med. **118**:766–770.