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Adsorption of Immunoglobulin A onto Oral Bacteria In Vivo

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Oral bacteria become coated with immunoglobulin A in human saliva in vivo. This may indicate that the salivary immunoglobulin A possesses antibacterial activity. Some of the immunoglobulin-coated cocci grow in extremely long chains and exhibit synchronous cell division. The long chain phenomenon may result from growth in the presence of salivary antibody specific to antigenic determinants of the bacterial cell walls.

The occurrence of antibacterial antibodies in human saliva has been reported by several investigators (for review, *see* 24, 25). The activities described were probably exhibited by immunoglobulin A (IgA), which is the major immunoglobulin of saliva (8, 12). There is, however, no direct evidence to support this assumption, although it has been shown that salivary IgA may carry activities against blood-group substances (36), intrinsic factor (9), poliovirus (4), and *Candida albicans* (11). Moreover, the IgA of colostrum and nasal secretions may exhibit both antiviral (2, 3) and antibacterial activities (1, 30). Therefore, it was of interest to determine whether IgA is adsorbed onto indigenous oral bacteria in vivo, in accordance with its postulated antibacterial function in external secretions (36).

MATERIALS AND METHODS

Smears of oral bacteria. Whole saliva was obtained without secretory stimulation from two children (both 12 years old) and one adult (30 years old) who were orally healthy and had normal amounts of immunoglobulins in their serum and saliva. The average salivary concentrations of IgA and immunoglobulin G (IgG) were 22.8 and 0.5 mg/100 ml, respectively, based on determinations with single radial immunodiffusion (8). Immunoglobulin M (IgM) could hardly be detected in the unconcentrated saliva with this technique. Whole saliva was similarly obtained from a hypogammaglobulinemic patient (15 years old) whose secretions were deficient in IgA. Supragingival dental plaque was collected from a normal adult.

Sediments of oral bacteria were obtained by collecting samples of dental plaque or unstimulated whole saliva into ice-cold isotonic phosphate buffer (PBS), *p*H 7.4, containing 0.01% Merthiolate, and subsequently centrifuging the suspensions at 10,000 × *g* and 4 C for 15 min. The sediments were washed three times in cold PBS, and finally heavy suspensions were made in the buffer. Before being washed, some samples were subjected to vibration in an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., London, England) operated at 1 amp for 1 min. Thin smears of the suspensions were dried at 37 C for 30 min and were fixed in 95% ethyl alcohol at 4 C for 30 min. When smears could not be made immediately, the bacterial suspensions were kept in small portions at −25 C until needed.

Cultures of oral bacteria. Mixed cultures of oral bacteria were grown in a broth medium consisting of 1.70% (w/v) Trypticase (BBL), 0.25% (w/v) yeast extract (Difco), 0.10% (w/v) casein (E. Merck), 0.50% (w/v) NaCl, 0.25% (w/v) K_2HPO_4, and 0.005% (w/v) $MgSO_4 \cdot 7H_2O$. The medium was adjusted to *p*H 7.1 and was autoclaved at 120 C for 20 min. The inoculates were derived from the gingival margins of four patients with gingivitis. The cultures remained at 37 C without stirring for 48 hr. The bacteria were then harvested by centrifugation and washed three times in PBS. The sediment contained gram-positive single cocci, diplococci, and streptococci in short chains. and gram-negative single cocci, short rods, and fusiform bacteria. It was enriched in gram-negative species by addition of an approximately equal volume of oral *Veillonella* sediment obtained as previously reported (17). Smears of this final mixture of cultivated oral bacteria were prepared as described for the salivary sediments.

Human immunoglobulins. IgG was a Cohn Fr. II preparation (Nutritional Biochemicals Corp., Cleveland, Ohio) further purified by diethylaminoethylcellulose chromatography (34). Pepsin-split IgG [F-$(ab')_2$] was prepared as described by Harboe (20). The digestion was carried out at 37 C and *p*H 4.0 for 24 hr with an enzyme to protein ratio of 2:100 (w/w). IgA was isolated from pooled human parotid secretion by the method of Tomasi et al. (36), and from human colostrum by the method of Cebra and Robbins (10). The 19*S* fraction of a human serum deficient in IgA was used as an IgM preparation devoid of other immunoglobulins. The fraction was obtained by centri-

fugation in a 10 to 40% sucrose gradient for 18 hr at 35,000 rev/min in a Spinco L/50 ultracentrifuge with an SW50 rotor.

Concentrations of immunoglobulin preparations were determined by single radial immunodiffusion as previously described (8). Concentrations of $F(ab')_2$ preparations in PBS were calculated from optical density at 280 mμ, with an $E^{1\%}_{1cm}$ of 13.3 (35).

Antisera. Monospecific rabbit antisera to the heavy chains of IgG, IgA, and IgM were prepared as reported elsewhere (8). A goat antiserum to IgA was purchased from Hyland Laboratories, Los Angeles, Calif. An antiserum to washed salivary sediment was obtained in the following way. Whole saliva was collected from three normal individuals as outlined above. The bacterial sediments were washed twice in PBS by stirring at 4 C overnight, and three times by centrifugation at 10,000 × *g* for 15 min. The final sediment was resuspended in PBS to a concentration of 13% (w/v), and was subjected to disintegration in an ultrasonic oscillator (Measuring & Scientific Equipment, Ltd.) operated at resonance frequency and maximal efficiency for ten 1-min periods, with interruptions to ensure effective cooling. The rabbit was first injected intramuscularly and subcutaneously with 3 ml of this suspension mixed with an equal volume of Freund's complete adjuvant (Difco). Two months later, the animal received 0.5, 1.0, and 1.5 ml of a similar suspension intravenously at intervals of 3 days. Blood was drawn 2 and 3 weeks after the last injection. To increase the titer of the antiserum, it was concentrated four times with dried acrylamide cylinders (15). To remove activities against the common determinants of immunoglobulin light chains, a portion of the concentrated antiserum was adsorbed with $F(ab')_2$ at a ratio of 0.82 mg/ml.

Immunofluorescence technique. Direct immunofluorescence tracing of IgA was performed on the bacterial smears. A goat antiserum to human IgA was employed (Hyland Laboratories); the IgG fraction of the antiserum was conjugated with fluorescein isothiocyanate (FITC). When the conjugated protein had been separated from the unreacted FITC by filtration through Sephadex G-25, the ratio of its optical densities in PBS at 280 and 495 mμ was 1.1:1. This relatively high degree of conjugation was found to be favorable, since the fluorescent features to be revealed were of low intensity.

The conjugate was applied to the smears in a concentration of 0.5 "unit" (6) of precipitating antibody per ml; the incubation period was 20 min at room temperature. Further details of the technique, as well as the adsorption and blocking controls, have been described elsewhere (21). Photographs were taken with Kodak Tri-X black-and-white film (27 Din).

The conjugate produced fluorescence of several types of bacteria in the smears of bacterial cultures, probably because of naturally occurring goat antibacterial antibodies (5). This fluorescence was abolished by repeated adsorptions of the conjugate with pooled sediments of mixed cultures and *Veillonella*.

Double diffusion and immunoelectrophoresis. Double diffusion and immunoelectrophoresis were carried out as previously described (7).

RESULTS AND DISCUSSION

Smears of salivary sediments and dental plaque exhibited the following features after exposure to the conjugate adsorbed with mixed cultures and *Veillonella*: bacteria with rims of green fluorescence (Fig. 1A and 3B); single or double long chains of cocci with a series of fluorescent green bands separated by nonfluorescent gaps (Fig. 1B and 2B); scattered, intensely green rod-shaped or oval bodies; epithelial cells with faint cytoplasmic and intense nuclear *auto*fluorescence (Fig. 2B and 3D); and diffusely green leukocytes ("salivary corpuscles") (31) with superimposed fluorescent bacteria and rod-shaped bodies (Fig. 2C).

The following criteria indicated that the bacterial fluorescence revealed as green rims or bands represented IgA. (i) The fluorescence was inhibited by adsorption of the labeled antiserum with IgA in excess, but not by adsorption with IgG (Fig. 2B, C); by prior application of an unlabeled rabbit anti-IgA to the smears, but not by the use of the preimmunization serum from the same rabbit; and by prior application of an unlabeled goat anti-IgA, but not by the use of a normal goat serum. (ii) Salivary sediments from the patient deficient in IgA did not exhibit bacterial fluorescence (Fig. 3C, D). (iii) Smears of mixed cultures of oral bacteria and *Veillonella* did not fluoresce.

Additional information was provided by immunochemical analyses with the antiserum to washed salivary bacterial sediment (Fig. 4 and 5). After adsorption with $F(ab')_2$, the antiserum still reacted with IgA, but not with IgM; remaining activity against IgG could hardly be detected. This indicated that the immune system of the rabbit had recognized IgA as the principal immunoglobulin present in the injected antigen preparation. In addition, the antiserum exhibited activities against albumin, amylase, and several bacterial antigens.

The fluorescence of the rod-shaped or oval bodies, scattered throughout the smears as well as within the leukocytes, did not fulfill the criteria of immunological specificity (Fig. 2C). These bodies probably represented leukocyte granules, since granulocytes are generally prone to particularly bright nonspecific staining (28). The bacterial fluorescence within the leukocytes was substantially, but not completely, inhibited in the adsorption and blocking tests, possibly because of superimposed nonspecific staining of the leukocytes.

The bacteria with fluorescent rims were mainly diplococci, but were also cocci or small rods, single or in short chains (Fig. 1A and 3B). The

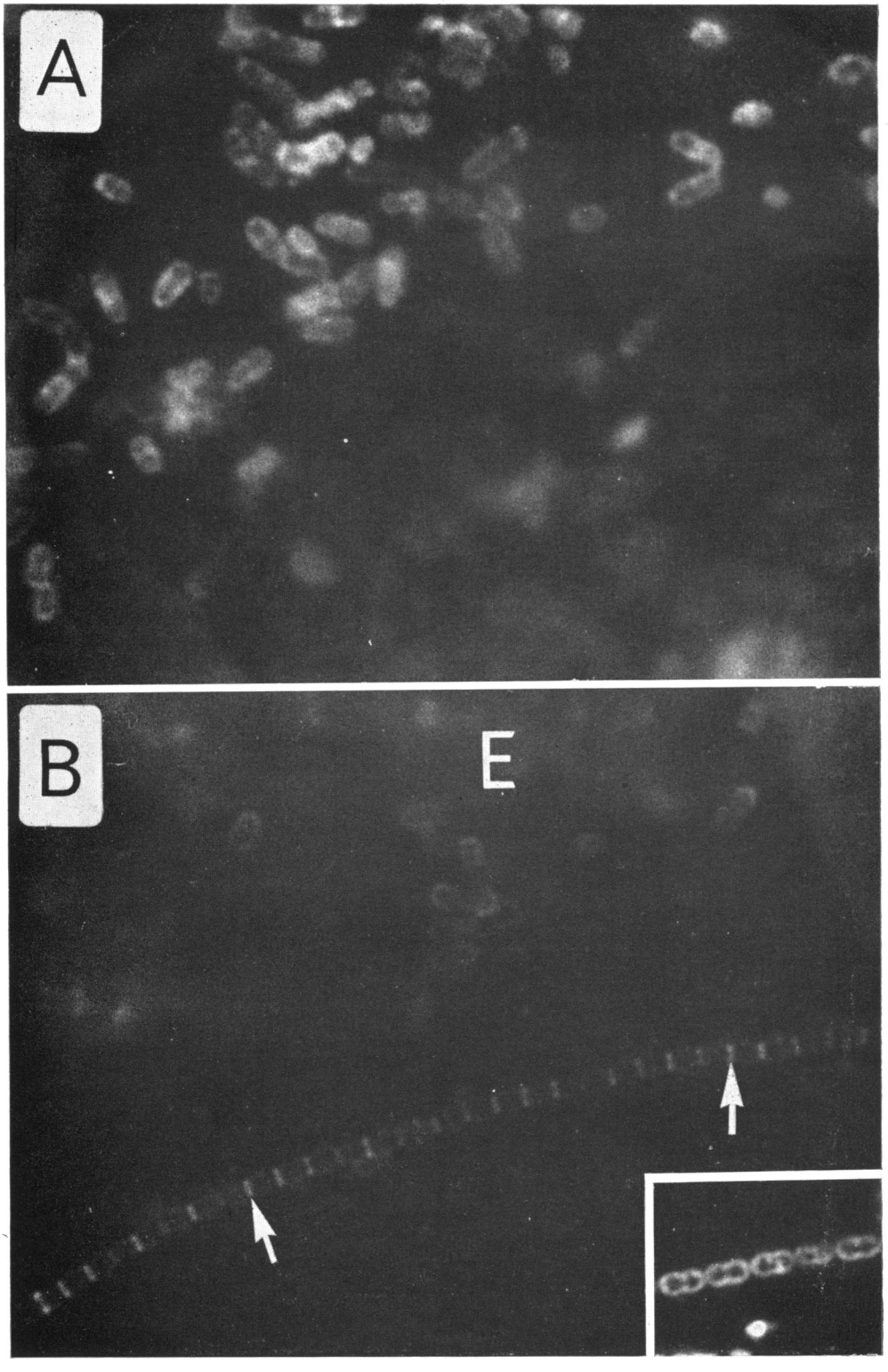

FIG. 1. *Smear of washed salivary sediment observed by fluorescence microscopy after reaction with fluorescein isothiocyanate-labeled anti-IgA. (A) Thin fluorescent rims around diplococci and cocci in short chains. (B) A single long chain of cocci with fluorescent bands (arrows). Insert, part of the chain observed in conventional dark field. E, epithelial cell covered with several fluorescent bacteria. × 3,700.*

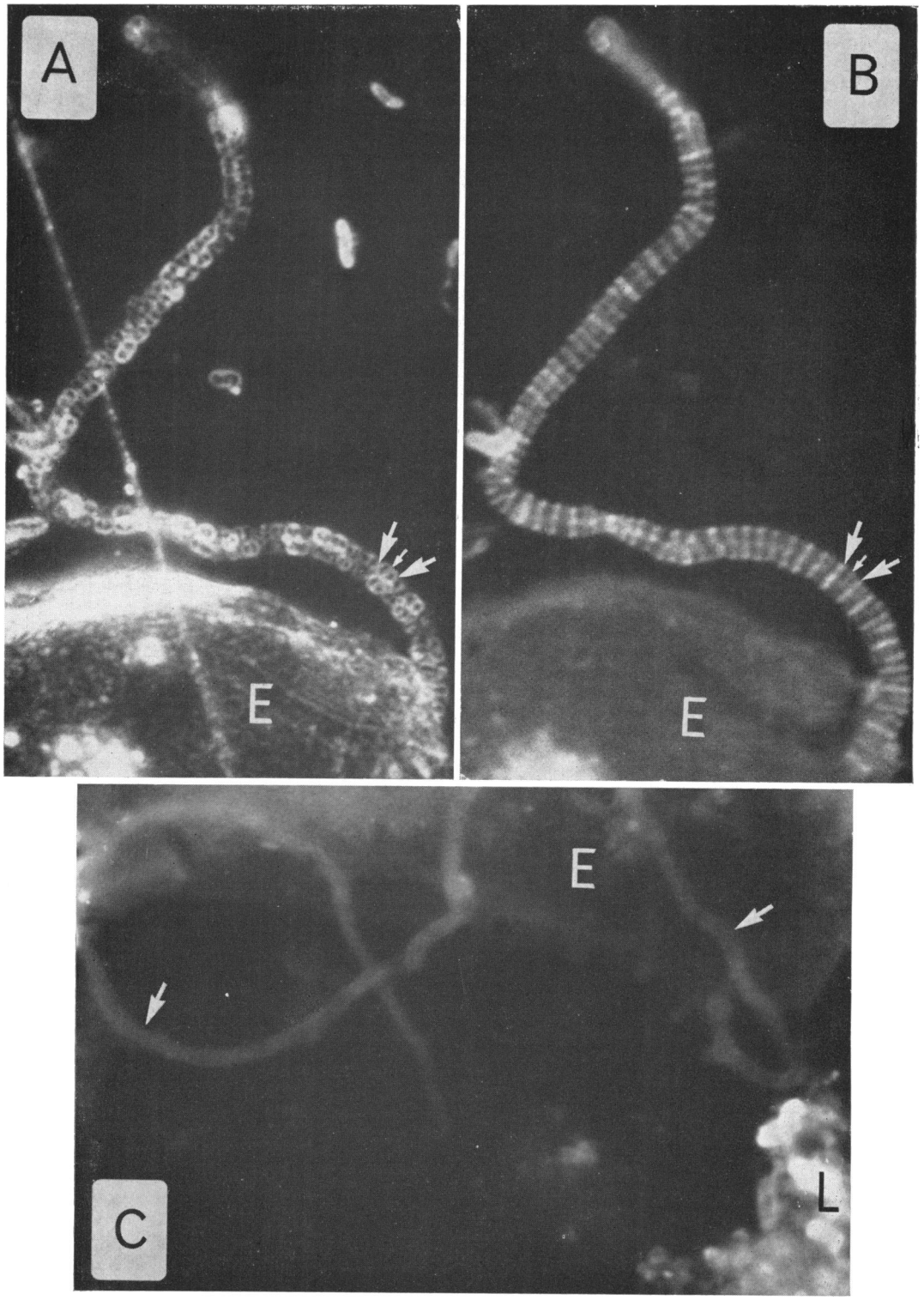

FIG. 2. *Smears of washed salivary sediments. (A) A long double chain of cocci adjacent to an epithelial cell (E) observed in conventional dark field. (B) The same chain observed by fluorescence microscopy. The smear had been exposed to fluorescein isothiocyanate-labeled anti-IgA adsorbed with IgG. Note specific fluorescence of older portions of cell walls (large arrows), and the relatively nonfluorescent younger portions including the cross walls (small arrows). The epithelial cell (E) is visualized because of autofluorescence. (C) A long single chain of cocci (arrows) and an epithelial cell (E) visualized because of autofluorescence. The smear had been exposed to fluorescein isothiocyanate-labeled anti-IgA adsorbed in excess with IgA. There was no specific immunofluorescence. Note nonspecific fluorescence of leukocyte (L).* × *2,600.*

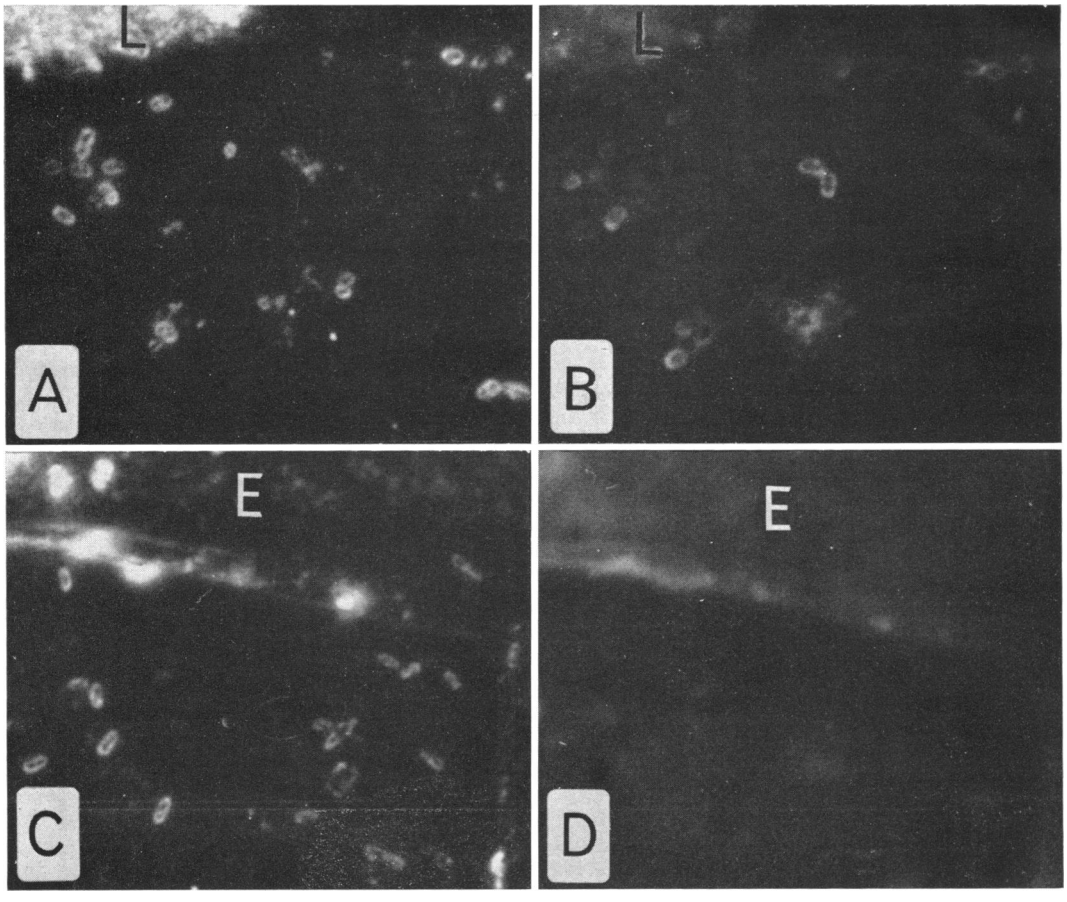

FIG. 3. *Smears of washed salivary sediments exposed to fluorescein isothiocyanate-labeled anti-IgA adsorbed with IgG. (A and C) Conventional dark field. (B and D) Fluorescence microscopy. L, leukocyte; E, epithelial cell. A–B is a smear from a normal individual; note that not all of the bacteria observed by conventional dark field exhibited immunofluorescence. C-D represents a smear from a patient deficient in IgA; note that none of the bacteria observed by conventional dark field exhibited immunofluorescence.* × *2,400.*

fluorescent bacteria covered the epithelial cells or were scattered between them. When a field was first observed in conventional dark field and then by fluorescence microscopy, it was apparent that some of the bacteria in the field did not exhibit fluorescence for IgA (Fig. 3A, B).

Extremely long single or double chains with fluorescent bands were observed in smears of salivary sediments from one of the children and, particularly, from the adult. The chains consisted of gram-positive cocci. The fluorescent bands were found to conform to the areas of end-to-end contact between the older portions of the cell walls (Fig. 2A, B). The younger portions of the walls, including the newly formed cross walls, exhibited little or no fluorescence. After ultrasonic treatment, the intensity of the fluorescence was unchanged, but the long chains were reduced to shorter ones.

The segmental fluorescence pattern apparently was a result of synchronous cell divisions within the bacterial chains, and may be explained in different ways. (i) Assuming that the supply of actual antibacterial IgA-antibody to the saliva was limited, only the antigenic groups of the older portions of the cell walls would be saturated with IgA at the time of sampling. Consequently, after reaction with labeled anti-IgA, the younger portions of the cell walls would appear relatively nonfluorescent. (ii) Assuming that the cocci in the chain were completely coated with IgA at the time of sampling, synchronous cell division must have occurred during or after the washing of the bacterial sediments. The exposure

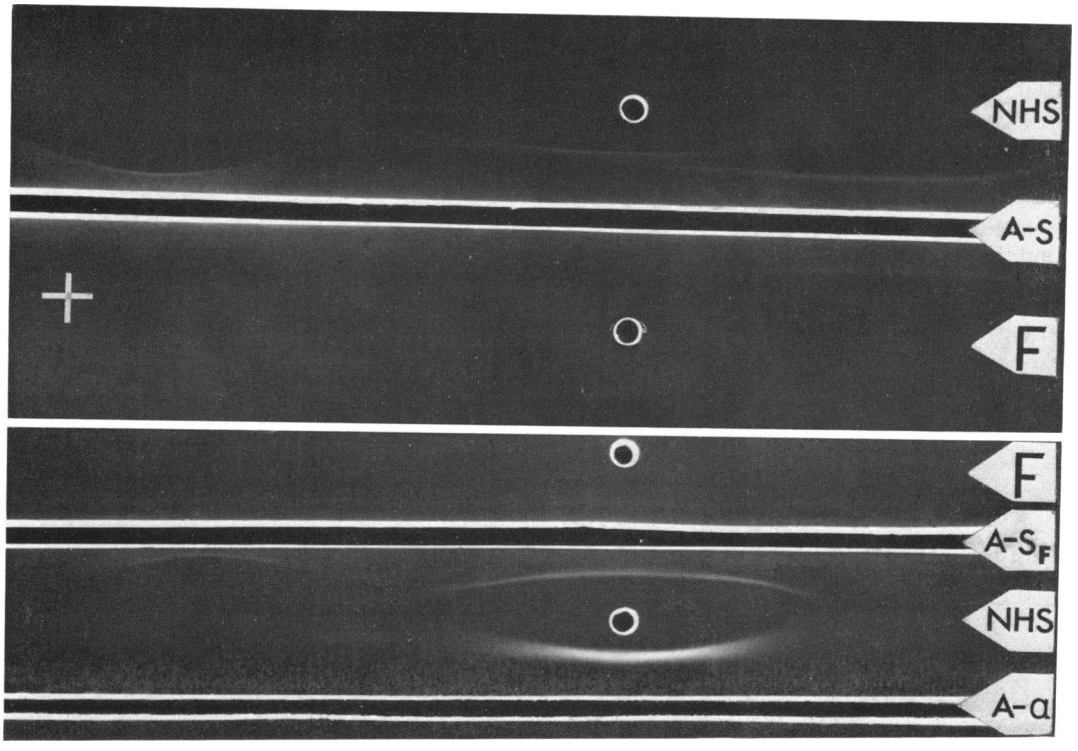

FIG. 4. *Antiserum to bacteria from human saliva examined for activities against immunoglobulins. Immunoelectrophoresis demonstrating that the antiserum (above) exhibited activities against F(ab')$_2$, I$_S$G, IgA, and albumin. After removal of activities against common antigenic determinants of immunoglobulins by adsorption with F(ab')$_2$, the antiserum (below) produced detectable precipitin arcs only with IgA and albumin. Antigens: NHS, normal human serum diluted 1:4; F, F(ab')$_2$ (0.2 mg/ml) obtained by pepsin digestion of human IgG. Antisera: A-S, antiserum to washed salivary bacterial sediment; A-S$_F$, the same antiserum adsorbed with F(ab')$_2$; A-α, antiserum to α-chain (specific anti-IgA).*

of the newly formed portions of the cell walls to free IgA would hence be reduced and finally eliminated through the washing procedure. The fluorescent bands would then represent cell wall formed in the saliva in vivo, whereas the relatively nonfluorescent segments would represent cell wall formed in vitro during or after, or during and after, the washing procedure. Thus, the result would be comparable to that obtained in immunofluorescence studies of bacterial cell wall replication (14, 37). The latter explanation seems plausible, since Cole (13) in such studies, due to the high sensitivity of the technique, was able to detect small amounts of bacterial growth in saline in the course of a few minutes.

It is not clear from our experiments whether IgA is adsorbed onto oral bacteria in vivo because of antibody activity, or because of the great tendency of IgA molecules to become "nonspecifically" conjugated with other protein molecules (22). Preliminary observations indicated that the adsorbed IgA could be eluted at *p*H 2.3. However, affinities other than antigen-antibody bindings may also be affected at this *p*H. The long-chain phenomenon exhibited by the gram-positive cocci might indicate that they had been growing in a milieu containing specific antibody. It is well known that the long-chain phenomenon may occur when bacteria are cultivated in antibody-containing artificial media (16, 19, 32, 33). The formation of long chains when *Streptococcus lactis* grows in raw milk (27) is also ascribed to an antibody effect (29). However, some types of oral streptococci (AHT, BHT, CHT, and KI-R) may grow in long chains even in a medium completely devoid of antibody (R. J. Fitzgerald, *personal communication*).

The possible biological significance of the coating of indigenous oral bacteria with IgA in vivo remains obscure. In addition, it is generally unknown how antibacterial antibodies function in external secretions in vivo. The antibody coat-

ing may enhance phagocytosis; our findings indicated that at least some of the bacteria engulfed by the "salivary corpuscles" were coated with IgA. Phagocytosis and the long-chain phenomenon may physically contribute to keeping the indigenous bacteria innocuous, and thus may be involved in host resistance. It also seems pertinent that the bacteriolytic factor occurring in the salivae of individuals immune to dental caries is found in the IgA fraction (18), and, furthermore, that individuals relatively prone to dental caries may have decreased concentrations of salivary IgA (26).

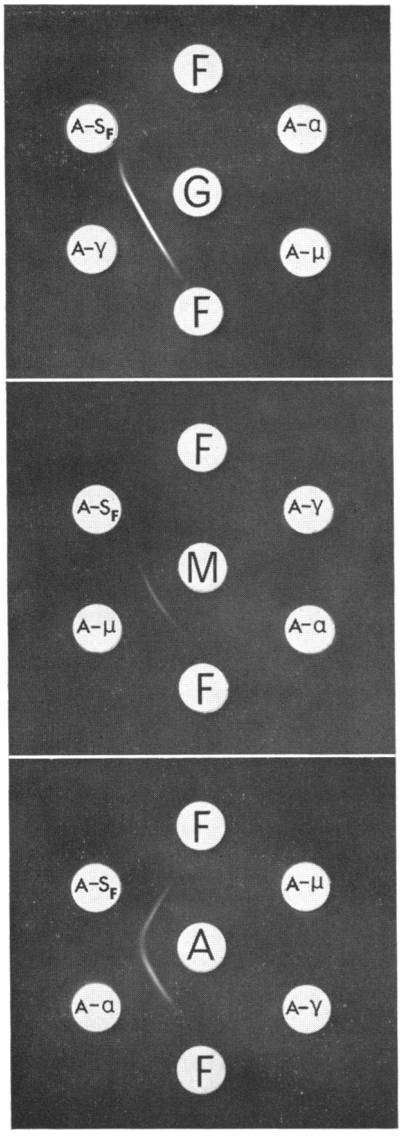

FIG. 5. *Antiserum to bacteria from human saliva examined for activities against immunoglobulins. Double-diffusion analyses revealing a distinct precipitin line with IgA, no reaction with IgM, and a hardly detectable reaction with IgG. Antigens: F, F(ab')$_2$ obtained by pepsin digestion of human IgG (0.2 mg/ml); G, human IgG (0.2 mg/ml); M, human IgM (0.2 mg/ml); A, human parotid IgA (0.2 mg/ml). Antisera: A-S$_F$, antiserum to washed salivary bacterial sediments adsorbed with F(ab')$_2$; A-γ, antiserum to γ-chain (specific anti-IgG); A-μ, antiserum to μ-chain (specific anti-IgM); A-α, antiserum to α-chain (specific anti-IgA).*

Antibodies of the secretory IgA-type may be better able to function in external secretions than those of the other immunoglobulin types. This was indicated by a recent study (23) on human milk antibodies to *Escherichia coli* and poliovirus. The milk antibodies were more resistant to acid and pepsin than were comparable antibodies in the serum. The IgA occurring in milk and other external secretions differs from that of serum in that it contains an additional protein portion called the "transport piece" or the "secretory piece" (8, 36). The ability of the secretory IgA to function as an antibody in the secretions may be due to this portion of the composite molecule, or to particular properties of the heavy polypeptide chain of IgA.

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