

ELECTRON MICROSCOPIC STUDIES ON STREPTOCOCCI

I. M ANTIGEN*

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(Received for publication 3 July 1969)

Extensive chemical and immunological studies have characterized the group A streptococcal cell wall as composed of four major components: M protein (and related T and R antigens), carbohydrate, mucopeptide, and glycerol teichoic acid (1, 2). These cell wall constituents, along with an external capsule and an underlying cytoplasmic membrane, are important in determining the relationship between group A streptococci and their human hosts (3). Our aim is to define the morphology and location of each of the major components of the streptococcal cell wall. The first component we have studied is the M antigen or M protein, the topic of this report.

M protein plays a role in determining virulence of streptococci, inhibits phagocytosis of the organisms by leukocytes, and stimulates production of type-specific, protective antibodies by the animal host (4). These effects, together with other properties attributable to the protein, have suggested to previous investigators that the M antigen is on the exterior of group A streptococci (1, 4). Our electron microscopic findings substantiate this concept. We have found that the presence of M antigen is associated with hair-like fimbriae on the outer surfaces of several strains of group A streptococci. It appears that multiple M antigenic sites reside along the length of the fimbriae. R antigens may be associated with the same type of fimbriae. We have also obtained data suggesting the attachment of the M-associated fimbriae to the underlying portions of the cell wall. Finally, our studies suggest that M protein is not incorporated into the cell wall simultaneous to the synthesis of other portions of the wall.

* This work was supported, in part, by United States Public Health Service grants HE 03929 (to Dr. Hsu) and HE 03919.

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Materials and Methods

Streptococci.—The various group A streptococci selected and used in this study were obtained through the generosity and with the advice of Dr. Rebecca Lancefield of The Rockefeller University. The organisms were examined for the presence of protein antigens by Dr. Lancefield (5-8).

In the first part of our study, streptococci to be examined were selected as pairs of strains, one of which had M antigen and the other of which had no detectable M. Each pair was derived from a common progenitor strain. The paired strains were used to avoid differences in wall composition, not related to M antigen, that might complicate comparisons in morphology. The pairs which were selected are listed below along with their type designations.

Type 6 S 43/100/15 (M+)

S 43 MA (M-)

Type 12 T 12/126/3 (M+)

T 12 gl (M-)

Type 1 T 1/195/1 (M+T+)

T 1 (M-T+)

Type 28 T 28/105A/2 (M+R+)

T 28/72/3 (M-R+)

Type 14 S 23/101/7 (M+)

S 23 Burbank (see Results for explanation)

These organisms were obtained as lyophilized specimens, were grown overnight at 37°C in Todd-Hewitt broth (9), and were stored in the frozen state for use as seed cultures. The frozen samples were thawed and inoculated into Todd-Hewitt broth in which the organisms were grown for 4-6 hr at 37° C prior to their use in morphological studies.

Trypsin Treatment and Reincubation.—An overnight culture was transferred to fresh Todd-Hewitt broth that contained 0.1% trypsin (Worthington Biochemical Corp., Freehold, N.J.). After 4-5 hr growth in this trypsin-containing broth (37° C), the cells were centrifuged, washed in phosphate-buffered saline (PBS) (Grand Island Biological Co., Grand Island, N.Y.) that contained 0.05% trypsin, and subsequently washed twice in trypsin-free PBS. The washed, centrifuged cells were suspended in prewarmed Todd-Hewitt broth not containing trypsin and were incubated at 37° C. Samples were removed for electron microscopic examination after 10 min, 30 min, and 60 min reincubation. Control specimens were also collected before and after trypsin treatment and washing. The specimens were fixed and processed as described below.

Ferritin-Conjugated Type-Specific Antibodies.—Type-specific antiserum to strain T 5B (type 5) streptococci was prepared in rabbits according to the method of Lancefield (8). The rabbit antiserum was absorbed with five heterologous types of group A streptococci to ensure minimal reactivity with cell wall components other than M protein. Globulins were precipitated from the absorbed antiserum by ammonium sulfate treatment (50% saturation). The precipitated globulin preparation was washed with and dialyzed against PBS until free of sulfate. The final volume of the globulin preparation was one-third the original volume of antiserum used. Ferritin conjugation of the globulin preparation was carried out by the method of Singer (10) as modified by Rifkind, Hsu, and Zabriskie (11). The final concentration of the ferritin-conjugated antibody preparation was 38 mg/ml.

Extraction of Group-Specific Polysaccharide with Nitrous Acid.—Overnight cultures of strain T 5B and of strain T 12/126/3 streptococci in Todd-Hewitt broth were exposed to nitrous acid in order to extract group-specific polysaccharide from their walls.¹ Rhamnose determinations on the whole cells both before and after this extraction were performed according to the method of Dische and Shettles (12). The nitrous acid-extracted and unextracted control cells were washed several times with water prior to fixation for electron microscopy.

¹ Gotschlich, E. C. Manuscript in preparation.

Electron Microscopy.—All streptococci except those exposed to ferritin-conjugated antibodies were treated in the following manner: Primary fixation was with 2% glutaraldehyde (Biological Grade, Fisher Scientific Co., Springfield, N.J.), in 0.1 M sodium cacodylate (K & K Chemical Co., Plainview, N.Y.), pH 6.8 for varying periods (4–16 hr). The fixed cells were washed several times with 0.1 M sodium cacodylate and treated for 1 hr with 1% osmium tetroxide in veronal acetate, pH 6.0 (13). After rinsing in veronal acetate buffer, the cells were embedded in 3–4% aqueous agar. The solidified agar was cut into small blocks, soaked in 1% uranyl acetate in veronal acetate buffer (final pH 4.5) for 1 hr, dehydrated in graded alcohol solutions, and embedded in Epon. Thin sections were stained either with lead citrate alone (14), or with 2% aqueous uranyl acetate (5 min), followed by water washing and lead staining (30 sec).

Streptococci which were to be exposed to ferritin-conjugated antibodies were fixed with 2% glutaraldehyde in PBS, pH 7.2 for 30 min and were rinsed several times with PBS. These fixed, washed, centrifuged cells (packed volume approximately 0.25 cc) were mixed with 0.25 ml of a solution of ferritin-conjugated antibodies (38 mg/ml) and allowed to stand for 30 min at 4° C. The cells were then washed five times with PBS and were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate for 30 min. After this step, the preparation followed the postfixation procedures outlined above. Electron micrographs were obtained with a Philips 200 instrument, which was used through the courtesy of Dr. Councilman Morgan.

RESULTS

General Comments on Morphology of Group A Streptococcal Cell Walls.—The appearance of the cell walls of intact group A streptococci varies somewhat with the mode of fixation. Primary osmium fixation, for example, provides clear demonstration of the cytoplasmic membrane but is associated with intensely electron opaque material on the surfaces of the streptococci. The opaque material is probably extraneous and obscures the external portions of the cell wall. Primary glutaraldehyde fixation, on the other hand, does not provide good visualization of the cytoplasmic membrane but yields reproducible demonstration of the cell wall. We have seen no essential difference between sodium cacodylate and PBS as buffer vehicles for glutaraldehyde.

The typical morphology for cell walls of glutaraldehyde-fixed group A streptococci can be seen in Fig. 1. The cytoplasmic membrane is indistinct. The cell wall consists of two distinct laminae, the inner of which is intensely electron opaque and is 80–100 Å thick. Peripheral to the intensely opaque layer is a thicker (120–150 Å), homogenous, compact zone which is of intermediate electron density. The thickness of this outer zone varies somewhat among strains of group A streptococci that we have studied. This variation is not found, however, in comparing the morphology of pairs of strains as shown below. Lamination of the homogenous, intermediate density zone can be demonstrated with the use of special electron stains² but is not present after fixation and staining procedures used in the present study. Neither is the capsule, so easily demonstrable by light microscopic study of India ink preparations, made visible by the methods employed here.

Comparative Morphology of M+ and M- Streptococci.—A consistent differ-

² Swanson, J., E. C. Gotschlich, and K. C. Hsu. Manuscript in preparation.

ence in morphology is found upon comparing M⁺ and M⁻ group A streptococci. Strains that lack M antigen (M⁻) have smooth, bare cell walls, whereas M⁺ strains have exteriors covered by hair-like fimbriae. This difference is seen upon comparing strain S 43 MA (M⁻) in Fig. 1 with strain S 43/100/15 (M⁺) in Fig. 2. These same relationships of the smooth exteriors of M⁻ strains and of the fimbriae-covered exteriors of M⁺ strains has been found with strain T 12 gl (M⁻, Fig. 3) and strain T 12/126/3 (M⁺, Fig. 4), as well as with strain T 1 (M⁻, Fig. 5) and strain T 1/195/1 (M⁺, Fig. 6). Other group A streptococci which were studied but which are not shown include both M⁺ (strain T 25/3) and M⁻ (strain J 17A4) organisms. These streptococci had fimbriae-covered and smooth, bare exteriors, respectively. Another culture of strain S 43/100 was examined in connection with a different study (15) and the cells were devoid of surface fimbriae. This finding was communicated to Dr. Maclyn McCarty who serologically tested the culture and could detect no M antigen.

Morphological differences were present in comparing strains T 1/195/1 and T 1, as mentioned above. These organisms differ in that only the former has M antigen. Both of these strains, however, have the T antigen. The absence of surface fimbriae on organisms which have T but lack M suggests that T antigen is not associated with surface fimbriae. On the other hand, if R antigen is present, surface fimbriae are observed regardless of whether M antigen is present (strain T 28/105A/2, M⁺R⁺, Fig. 8) or absent (strain T 28/72/3, M⁻R⁺, Fig. 7). This finding suggests either that M antigen and R antigen are present on the same fimbriae or else that each of these protein antigens is associated with separate, but morphologically similar fimbriae.

Strain S 23/101/7 and strain S 23 Burbank were chosen for study as another pair of M⁺ (type 14) and related M⁻ organisms, respectively. However, both the M⁺ (Fig. 9) and the supposed M⁻ (Fig. 10) streptococci are covered with fimbriae. Accordingly, the strain S 23 Burbank was retested and again found to lack type 14 M antigen. On the basis of our finding, it was speculated that a previously unrecognized M antigen might be present on strain S 23 Burbank streptococci. Dr. Grove Wiley of the Alfred I. duPont Institute has recently substantiated that speculation by demonstrating that type 51 M antibodies, as assayed in bactericidal and precipitin reactions, are produced in rabbits immunized with S 23 Burbank.³

The two compact laminae of the cell wall—the intensely electron opaque inner layer and the intermediate density outer layer—were carefully compared in studying the paired M⁺ and M⁻ strains. We have detected no differences in these compact layers that can be correlated with either the presence or the absence of M antigen.

Trypsin Treatment and Reincubation of M⁺ Streptococci.—Exposure of M⁺

³ Wiley, G. Personal communication.

streptococci to trypsin has been shown to result in removal and destruction of M antigen (16, 17). This treatment does not affect the viability of the streptococci which, upon washing and reincubation, in trypsin-free medium, regenerate M antigen. An experiment of trypsinization and reincubation of M+ streptococci (strain S 43/100/15) was carried out to evaluate the changes in cell wall structure that obtain with removal and reappearance of the M antigen.

The exterior surfaces of cells in the "control" specimen (fixed prior to trypsin treatment) are covered with fimbriae, as shown in Fig. 11. Incubation of the same culture with trypsin resulted in the expected changes in cell wall morphology. These cells (Fig. 12) are completely smooth and devoid of fimbriae. Reincubation of the trypsinized culture in trypsin-free, prewarmed broth is accompanied by prompt reappearance of surface fimbriae as shown in Figs. 13, 14, and 15. Small foci of "hair" are present on the surfaces of streptococci that have been reincubated for 10 min (Fig. 13). These foci are in the vicinity of cross walls or septa which are in the intermediate or late stages of development. After 30 min (Fig. 14) and 60 min (Fig. 15) reincubation, the streptococci are nearly completely covered with fimbriae. The short segments of cell wall surface that overlie cross-septa in the earliest stages of formation (as judged by their shallow contours) are often devoid of fimbriae. These foci and other parts of the wall on which fimbriae are sparse are seen better when reincubated streptococci are exposed to ferritin-conjugated antibodies (described later).

Localization of Ferritin-Conjugated Antibodies.—Antigens can be localized by electron microscopy through the use of ferritin-conjugated antibodies that provide electron opaque markers for the sites of antibody-antigen combinations (11). We have utilized ferritin-conjugated antibodies to localize M antigenic sites in relation to the fimbriae on the exterior of the M+ streptococcal walls.

Incubation of type 5 (strain T 5B, M+) streptococci with a ferritin-conjugated type 5 antibody preparation (see Materials and Methods) results in heavy tagging of the cells (Fig. 16). Ferritin is distributed several particles deep on the exterior of each homologous cell. In some foci, the ferritin particles have a linear arrangement that radiates from the compact portion of the underlying cell wall. This pattern mimics the form of the surface fimbriae. The number of ferritin particles in such a linear arrangement varies both with the thickness of the section and with the plane at which the cell wall surface is viewed, but is usually from three to five particles along a single radiation (see also Fig. 25). Ferritin particles are sparse or absent overlying the shallow invaginations of the cell wall that mark beginning septa formation (asterisks, Fig. 16).

Nonspecific ferritin tagging was evaluated by incubating type 6 (strain S 43/100/15, M+) streptococci with the same ferritin-conjugated antibody

preparation used in the above-described experiment. The results are shown in Fig. 17. Few ferritin particles are present on the surface fimbriae of these heterologous cells and are localized at the outermost tips of the fimbriae (arrows, Fig. 17). Dense accumulations and linear arrangements of ferritin particles are not seen on these type 6 cells exposed to the ferritin-conjugated type 5 antibodies.

Localization of Ferritin-Conjugated Antibodies on M+ Cells after Removal and Subsequent Regeneration of M Antigen.—Streptococci of strain T 5B that are treated with trypsin and then exposed to ferritin-conjugated type 5 antibodies do not have ferritin particles on their smooth, bare external surfaces (Fig. 19; compare with organisms before trypsin exposure, Fig. 18). Incubation of the cells in fresh, trypsin-free broth is accompanied by renewed ferritin tagging of the cell wall surfaces (Figs. 20–22). After 10 min reincubation (Figs. 20a and 20b), ferritin particles are located over developing cross-septa and in the crevices between nearly completely divided streptococcal pairs along a chain (Fig. 20a). By 30 min reincubation, the pattern of ferritin distribution indicates that septa, which were seen in early or intermediate stages of formation at 10 min, have almost been completed. This accounts for heavy ferritin-tagging of alternating pairs of apposed convex surfaces of streptococci along a chain (a, Fig. 21). The other parts of the streptococcal surface bear few ferritin particles (s, Fig. 21). After 60 min reincubation, the majority of streptococci in the specimen show heavy ferritin tagging of their cell wall surfaces (a, Fig. 22). Sparse distribution of ferritin is present on a few surfaces (s, Fig. 22). Ferritin particles are frequently absent overlying the segments of the cell wall, at which, beginning septum formation is present (asterisks, Figs. 21 and 22).

Appearance of M-Associated Fimbriae after Nitrous Acid Extraction of Cell Wall Polysaccharide.—One of us (E.C.G.) has found that a single extraction with nitrous acid of either isolated cell walls or of intact streptococci will remove most of the group specific carbohydrate. This extraction procedure reduces the rhamnose content of isolated walls from 19 mg/100 ml to 7 mg/100 ml. It has also been found that this comparatively gentle extraction does not result in solubilization of M antigen from M+ cell walls or from intact, whole M+ streptococci. Prior to nitrous acid treatment, strain T 12/126/3 (M+) streptococci have the cell wall architecture seen in Fig. 23. The surface fimbriae cover the homogenous, 130–150 Å thick zone of intermediate electron density that constitutes the outer compact portion of the cell wall. After nitrous acid extraction, surface fimbriae are still present and appear to be attached to the 65 Å thick cell wall that remains (Fig. 24). This 65 Å thick residual portion of the cell wall that is not solubilized by nitrous acid represents, primarily, mucopeptide and is derived from the 130–150 Å thick zone of intermediate electron density of control, unextracted cell walls.² The reduction in thickness from the original dimensions (130–150 Å) to 65 Å parallels the removal of the bulk of the

group specific carbohydrate. Of greater importance in the present study is the finding that, in spite of removal of most of this polysaccharide, the M-associated fimbriae are still in continuity with the residual portion of the cell wall. We have been unable to determine, to our satisfaction, whether the fimbriae are longer in extracted than in control, unextracted cells.

Localization of Ferritin-Conjugated Antibodies on Nitrous Acid-Extracted M+ Cells.—We were curious as to whether extraction of group specific polysaccharide from the cell wall would result in “new” M antigenic sites along the bases of fimbriae which had previously been “covered” by carbohydrate. In an attempt to answer this question, we exposed strain T 5B streptococci to ferritin-conjugated type 5 antibodies after the cells were extracted with nitrous acid. A similar exposure to ferritin-conjugated antibodies was carried out with control, unextracted strain T 5B cells. The comparative results are shown in Figs. 25 and 26. Ferritin particles are abundant and have linear distribution patterns (corresponding to radially arranged surface fimbriae) in both the control and the nitrous acid-extracted organisms (arrows, Figs. 25 and 26). Micrographs of both specimens were chosen for similarity of focus and section thickness and were enlarged to the same degree. Portions of the cell wall seen in sharp profile were analyzed by measuring the distance from each ferritin particle to the outermost, convex surface of the compact portion of the cell wall. These measurements are as follows: controls (before extraction), mean distance = 265 A; after extraction, mean distance = 325 A. The difference (325–265) is 65 A, about the same dimension as the portion of compact cell wall which was removed by nitrous acid extraction. This suggests that ferritin particles lie further from the outer surface of the compact wall after nitrous acid extraction than in control cells. The standard deviations on these data, however, are so large that tests for differences between the two means lack significance.

DISCUSSION

Several lines of indirect evidence have suggested to previous investigators that M antigen is located on or near the exterior of the streptococcal cell wall. The ability of M+ organisms to resist phagocytosis by leukocytes (review, reference 4), the easy removal of M antigen from viable streptococci (16, 17), and the pattern of immunofluorescent staining (18, 19) have all been interpreted as evidence that M protein is on the external surface of the cell wall. We have restudied the question of the location of M protein by electron microscopic methods that provide the resolution necessary for a more direct answer to this problem.

Morphological study of a dozen different strains of group A streptococci (both M+ and M–) suggest that the M antigen is associated with radially arranged surface fimbriae that extend outward from the underlying compact

portion of the cell wall. These fimbriae are present in every strain studied in which M antigen is demonstrable by established methods of extraction and assay. The presence or absence, respectively, of these fimbriae is the only difference in cell wall morphology that is visible in paired M+ and M- strains which are similar in other aspects of cell wall composition and structure. The surface fimbriae do not appear to be exclusively associated with M antigens. Strains of streptococci that have R antigen bear surface fimbriae on their cell walls, regardless of whether M is present or absent. This contrasts with T antigen, which does not appear to be associated with surface fimbriae.

In a previous electron microscopic study of streptococci (20), it was suggested that M protein occurs as a "microcapsule" on the surface of the organisms. While it seems probable that the material designated as microcapsule corresponds to the surface fimbriae described in the present report, the published micrographs do not permit direct comparison.

Visualization of the electron opaque markers of ferritin-conjugated type 5 antibodies attached to type 5 streptococci strongly suggests that M antigenic sites are located on the surface fimbriae. The linear pattern of ferritin particles on these homologous M+ cells corresponds to several M antigenic sites along the length of single fimbria whose direct visualization is compromised by the heavy ferritin tagging. The linear arrangement of ferritin particles does not result from nonspecific attachment and aggregation of ferritin-conjugated antibodies on the streptococcal surface. This is shown in type 6 controls which were incubated with ferritin-conjugated type 5 antibody and which do not show a linear arrangement of the few ferritin particles that are present. Ferritin tagging of these controls is confined to the tips of the surface fimbriae and probably results either from nonspecific "stickiness" of surface fimbriae or from the presence of unidentified antigens that are shared by the fimbriae on type 5 and type 6 organisms.

A recent report has dealt with ferritin-conjugated antibodies directed against M protein and visualized by electron microscopy (21). Ferritin is described as being "both on and within the relatively loose substance of the microcapsular layer". Examination of the accompanying micrograph (Fig. 8, ref. 21) shows sparse ferritin tagging in a seemingly dense, concentric lamina whose relationship to other portions of the cell wall is unclear. These findings are difficult to interpret.

The changes in cell wall morphology after trypsin treatment and subsequent reincubation support the concept that surface fimbriae are associated with M protein. It is well established that trypsin removes M protein from streptococci and destroys it as an antigen (16, 17). Under similar conditions of trypsin treatment, the surface fimbriae are completely removed and the localization of ferritin-conjugated M antibodies is eliminated. On reincubation of the organisms in trypsin-free broth, the fimbriae reappear and their antigenic sites

can again be demonstrated with ferritin-conjugated M antibodies. This parallels the reappearance of M protein demonstrated by classical methods of extraction and serological assay (8, 17).

After short term reincubation of trypsinized streptococci, the location of fimbriae and localization of ferritin-conjugated antibodies both follow, in general, the pattern of new cell wall synthesis as previously shown in experiments utilizing fluorescein-conjugated antibodies directed against streptococcal wall material (22). Our studies on long term reincubation, however, demonstrate exceptions in the case of M antigen to this general pattern of cell wall synthesis. First, prolonged reincubation of trypsin-treated streptococci in fresh broth results in distribution of fimbriae and ferritin tagging of two types. The majority of streptococci are covered by abundant fimbriae and heavy ferritin tagging. This resembles what is seen in nontrypsinized controls. The distribution of these heavily ferritin-tagged fimbriae roughly corresponds to the portions of cell wall that appear to be newly synthesized (22). The remaining segments of the streptococcal surfaces correspond to cell wall formed before the end of trypsin treatment, i.e., "old" cell wall. We find that these old portions of the cell wall bear few fimbriae and are lightly tagged by ferritin-conjugated antibodies. These features contrast sharply with the complete absence of surface fimbriae and the lack of ferritin localization on cells immediately after trypsin exposure. The findings suggest that M antigen is, in fact, present in small amounts on the old portions of the cell wall.

The second finding in this study which seems to differ from the general pattern of new cell wall synthesis concerns the relationship of fimbriae and ferritin tagging to septa that are in the earliest stages of formation. Several examples have been shown in which both surface fimbriae and ferritin-conjugated antibodies are absent on those parts of the wall that immediately overlie the shallow contours of beginning septa. It is assumed that both the portions of wall which invaginate to form a septum (or are synthesized in a centripetal direction) and the short segment of peripheral cell wall immediately continuous with the beginning septum are newly synthesized. If the full thickness of the cell wall (including M-associated fimbriae) were synthesized and assembled simultaneously, there should be no areas which are devoid of fimbriae and ferritin tagging. The bare foci, however, are present and suggest that full thickness synthesis and assembly of the cell wall (including M antigen) do not occur. In addition, we have seen no evidence of fimbriae or ferritin localization on those portions of the cell wall that constitute the intercellular septa that incompletely divide cells during the late stages of development (examples in Figs. 18, 20b, and 21). These findings all suggest that M protein is not incorporated into the cell wall simultaneously with the formation of the underlying, compact laminae of the wall. The observed data suggest that M protein is secreted by the cell, is partially excreted through the cell wall, and can be retained (or

bound) by the compact portions of the cell wall such that it (M antigen) is retained on the outer surface of the streptococcus. This concept does not fully explain the differential densities of fimbriae and ferritin tagging which are found on old portions of the cell wall as compared to newly synthesized segments after trypsinization. Factors such as differences in porosity between old and new segments of the cell wall, regions in the cell in which M protein is most actively synthesized, etc., may contribute to the patterns we have observed, but such factors are at present unknown. Answers to these questions can probably be reached through the simultaneous use of ferritin-conjugated anti-M antibodies and ferritin-conjugated anti-carbohydrate antibodies. These experiments are currently in progress.

The results of a previous immunofluorescent study (19) of the removal and reappearance of M protein appear, at first glance, to contradict the concept of M protein secretion by streptococci. Careful examination of the micrographs in that report, however, suggests that faint fluorescence is present on the cell wall segments that were present at the end of trypsin treatment and were not newly synthesized during subsequent growth in trypsin-free medium (Figs. 1e and 1f, ref. 19). Because of the equivocal faint staining in those old regions of the wall and because of the limited resolution of the light microscope, that previous report is not necessarily at variance with our suggestion that M protein is secreted through and becomes attached to the otherwise complete streptococcal cell wall.

We have shown that M-associated fimbriae are retained on M+ streptococci from whose walls the major part of group-specific polysaccharide has been extracted by nitrous acid. Therefore, it appears that the M-associated fimbriae are bound to the portion of the cell wall that is not solubilized by nitrous acid, namely mucopeptide and a minor fraction of group-specific polysaccharide. This agrees with observations on the products of enzymatic lysis of streptococcal cell walls (23). In those studies, two separable fractions that contained group-specific polysaccharide were obtained after lysis of isolated cell walls with phage-associated lysin. The major carbohydrate fraction was free from M protein and probably corresponds to that portion of carbohydrate solubilized by nitrous acid. The minor carbohydrate fraction was bound to M protein. This minor carbohydrate component, along with mucopeptide, probably represents the cell wall remaining after nitrous acid extraction in our study. Whether the M-associated fimbriae are attached to mucopeptide or to the remaining group-specific polysaccharide is not clear at present.

The surface fimbriae which remain attached to the nitrous acid-extracted streptococcal cell walls retain the ability to bind ferritin-conjugated anti-M antibodies along the length of the fimbriae. In spite of careful comparison of control and extracted cells that have been tagged with ferritin-conjugated antibodies, we have been unable to establish whether or not solubilization of the

bulk of group-specific polysaccharide "uncovers" M antigenic sites at the bases of the M-associated fimbriae. The mean distances between ferritin particles and the outer surfaces of cell walls and visual comparison of control and nitrous acid-extracted, ferritin-tagged cells gives the impression that the distances are greater in the extracted cells. This suggests that no new antigenic sites are uncovered by removal of polysaccharide from the bases of the surface fimbriae, but this impression is without clear proof.

SUMMARY

The presence of M antigens on group A streptococci is associated with hair-like fimbriae that cover the surface of the streptococcal cell wall and are demonstrable by electron microscopy. These fimbriae also may be associated with R antigen. Like M protein, the surface fimbriae are destroyed by trypsin treatment and reappear when "trypsinized" streptococci are reincubated in fresh, trypsin-free broth.

Ferritin-conjugated, type-specific antibodies localize on homologous M+ cells in a pattern suggestive of several M antigenic sites along the length of individual surface fimbria. The M-associated fimbriae remain on the residual cell wall after removal of the bulk of group-specific polysaccharide through nitrous acid extraction. This suggests attachment of the fimbriae to the mucopeptide and minor polysaccharide components remaining in the nitrous acid-extracted wall.

The pattern of localization of ferritin-conjugated antibodies on homologous streptococci before and after trypsin exposure and upon reincubation of the trypsinized cells in fresh medium suggests the following hypothesis: M antigen is secreted by the cell, is partially excreted through the otherwise intact cell wall, and is bound by the wall so that M protein occupies a peripheral, exposed position on the surfaces of the streptococcal cell wall.

The authors sincerely appreciate the advice and the helpful discussions of Dr. Rebecca Lancefield, Dr. Maelyn McCarty, and Dr. John Zabriskie during the course of this study. We also thank Dr. Stuart Elliott for his help in reviewing the manuscript.

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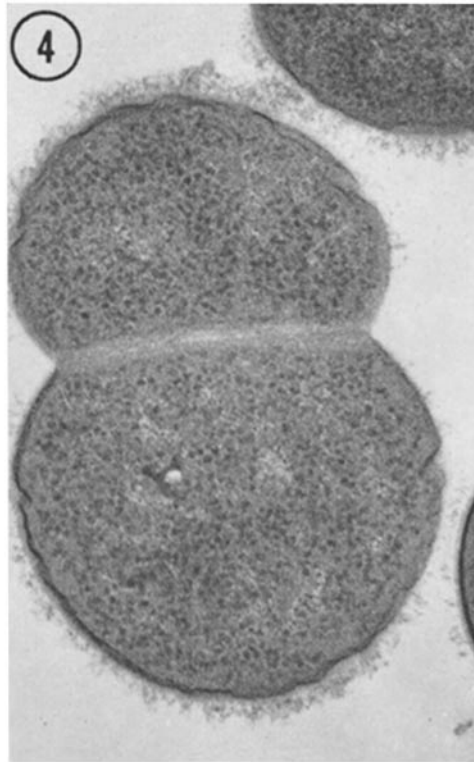
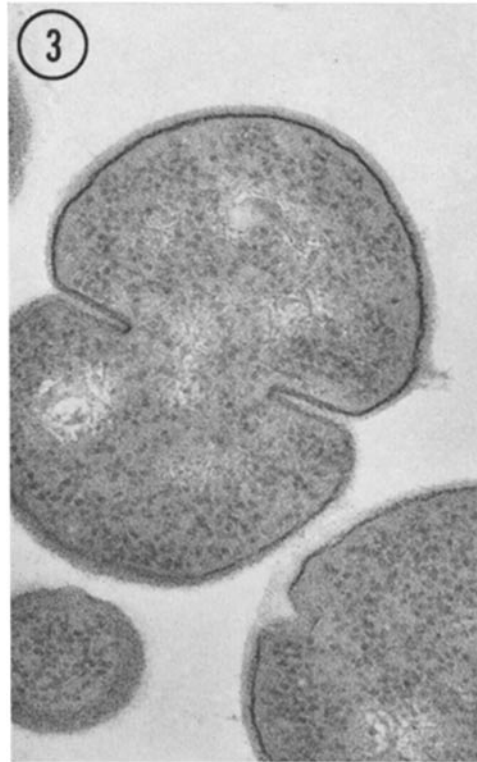
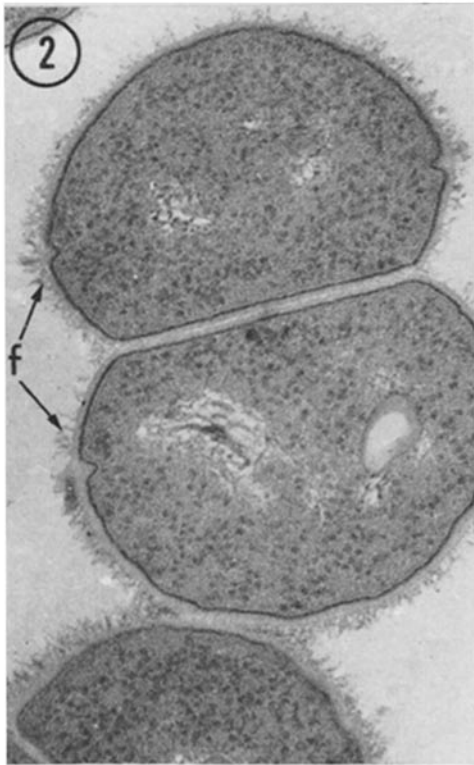
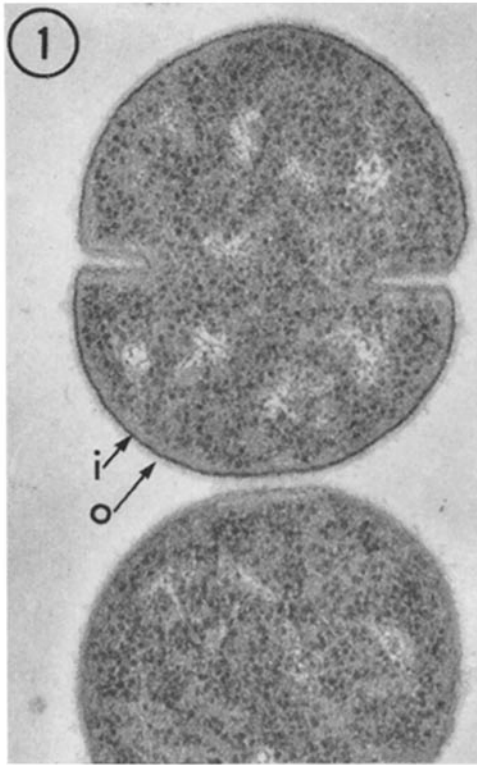
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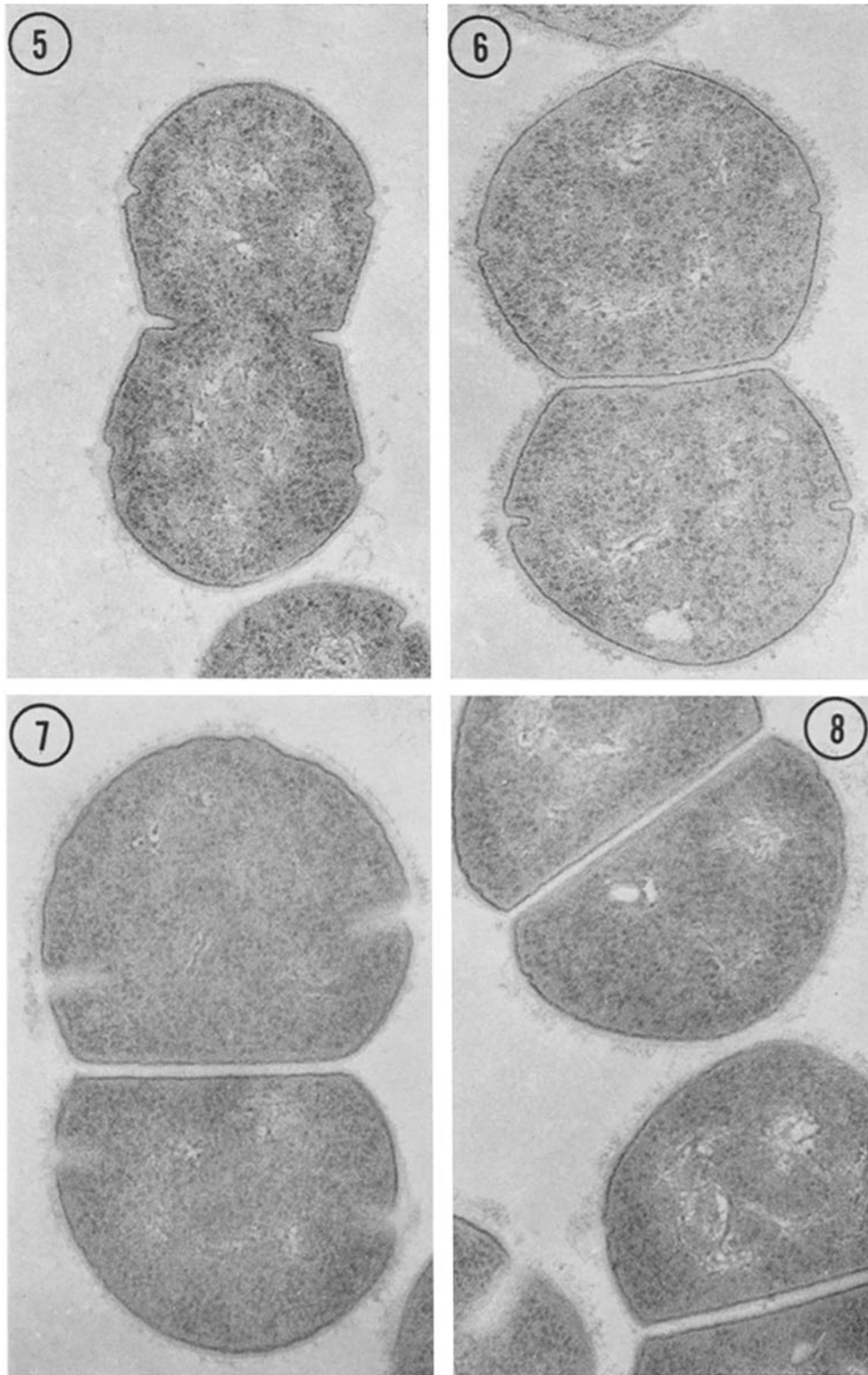
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FIG. 1. Strains S 43 MA (M-) streptococcal cell walls are composed of two compact laminae. The inner (i) layer is intensely electron opaque and lies outside the indistinct cytoplasmic membrane. The outer (o) compact layer is homogenous in appearance and has intermediate electron opacity. The external surfaces of the cells are smooth. $\times 60,000$.

FIG. 2. Strain S 43/100/15 (M+, type 6) streptococci have cell walls that are covered by hair-like fimbriae (f). The two compact laminae of the underlying cell wall are identical to those in similar (S 43 MA) M- organisms shown in Fig. 1. $\times 60,000$.

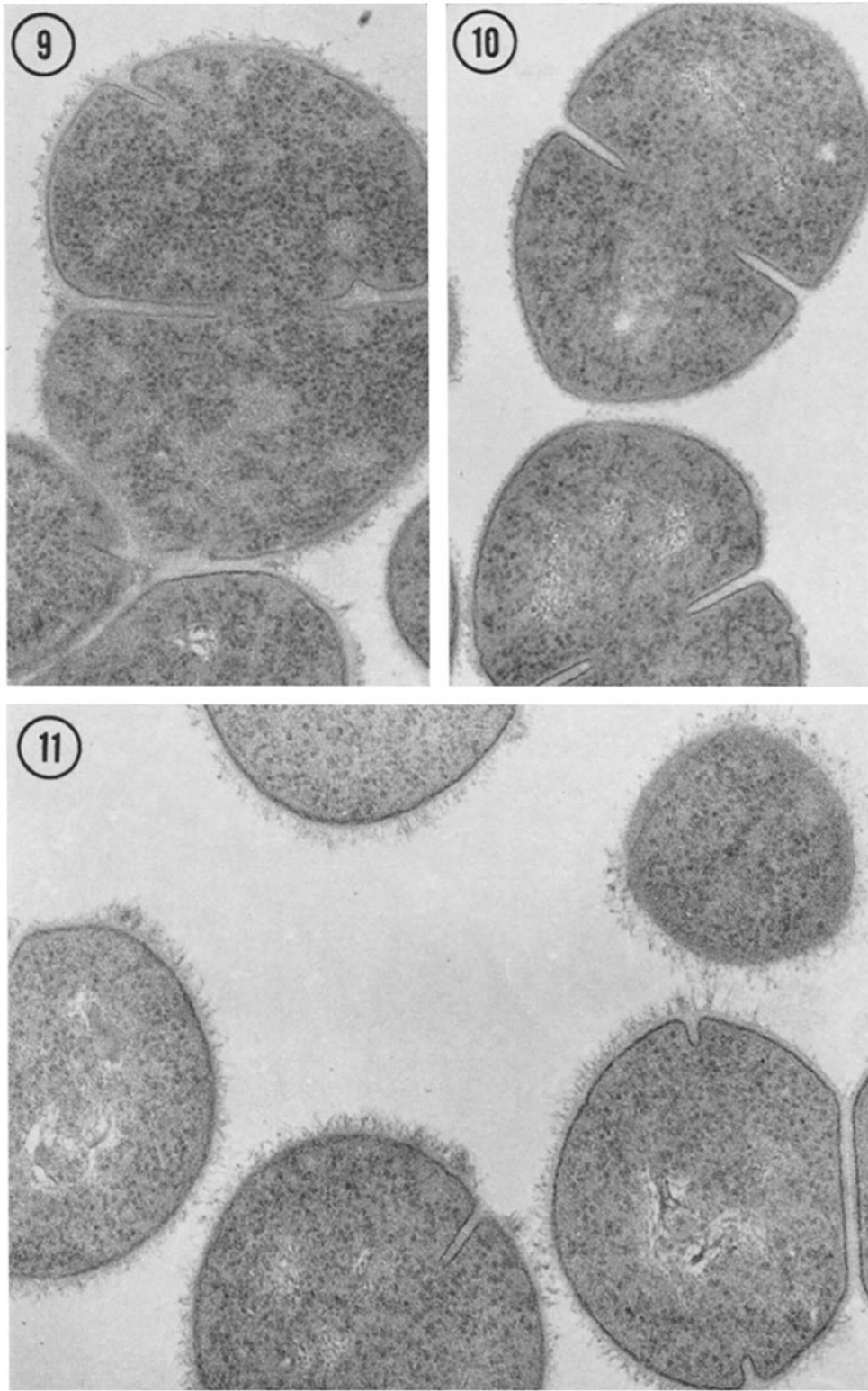
FIG. 3 and 4. Strain T 12 gl (M-) streptococci have smooth, bare exteriors (Fig. 3) whereas strain T 12/126/3 (M+) cells are covered by fimbriae (Fig. 4). $\times 60,000$.





FIGS. 5 and 6. Strain T 1 (M⁻, T⁺) organisms have smooth exteriors (Fig. 5). Similar M⁺ organisms of strain T 1/195/1 (M⁺, T⁺) are covered by fimbriae (Fig. 6). $\times 60,000$.

FIGS. 7 and 8. Strain T 28/72/3 (M⁻, R⁺) (Fig. 7) and strain T 28/105A/2 (M⁺, R⁺) (Fig. 8) both bear surface fimbriae. $\times 60,000$.



FIGS. 9 and 10. Strains S 23/101/7 (type 14, M+) (Fig. 9) streptococci are coated with surface fimbriae. Definite, short fimbriae are also present on streptococci of strain S 23 Burbank (type 14 M-, type 51 M+) (Fig. 10). $\times 60,000$.

FIG. 11. Control specimen from culture of strain S 43/100/15 fixed prior to trypsin exposure. Fimbriae coat the surfaces of these M+ cells. $\times 60,000$

FIG. 12. After trypsin treatment the streptococci of strain S 43/100/15 are completely devoid of surface fimbriae. $\times 60,000$.

FIG. 13. After 10 min reincubation in fresh broth the streptococci have fimbriae on their walls in the vicinities of developing septa. $\times 60,000$.

FIG. 14. Streptococci have abundant fimbriae on approximately one-half of their surfaces after 30 min reincubation in trypsin-free broth. The fimbriae coat the portions of the wall adjacent to septa that completely divide daughter cells. Other parts of the streptococci have scattered, scant surface fimbriae. $\times 60,000$.

FIG. 15. 1 hr reincubation results in coating of most streptococcal surfaces with fimbriae. The fimbriae are sparse or absent on cell wall exteriors where septa are beginning to form (*). $\times 60,000$

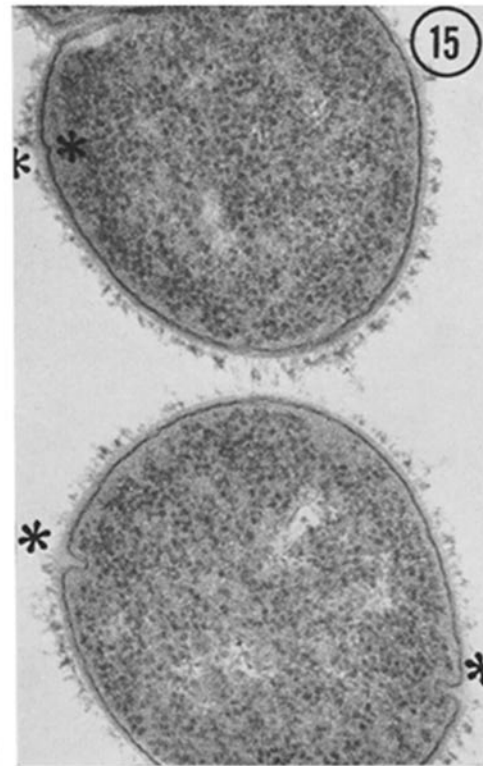
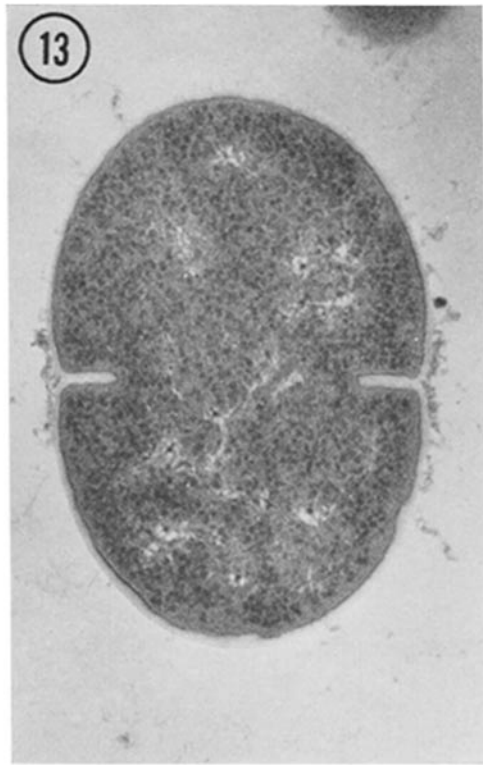
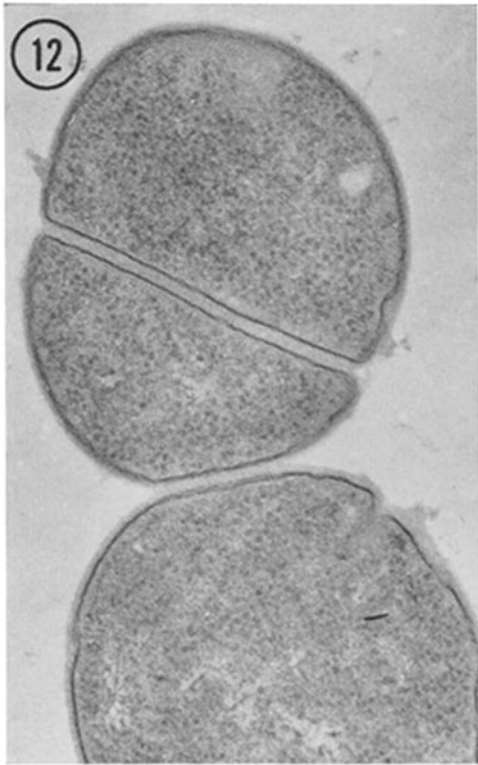
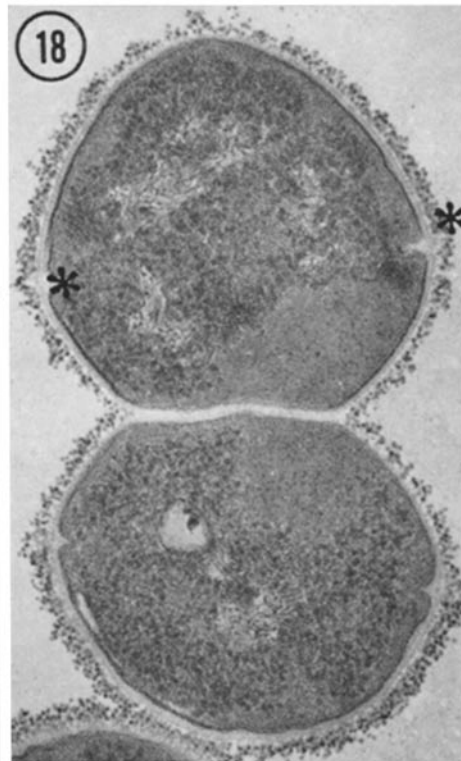
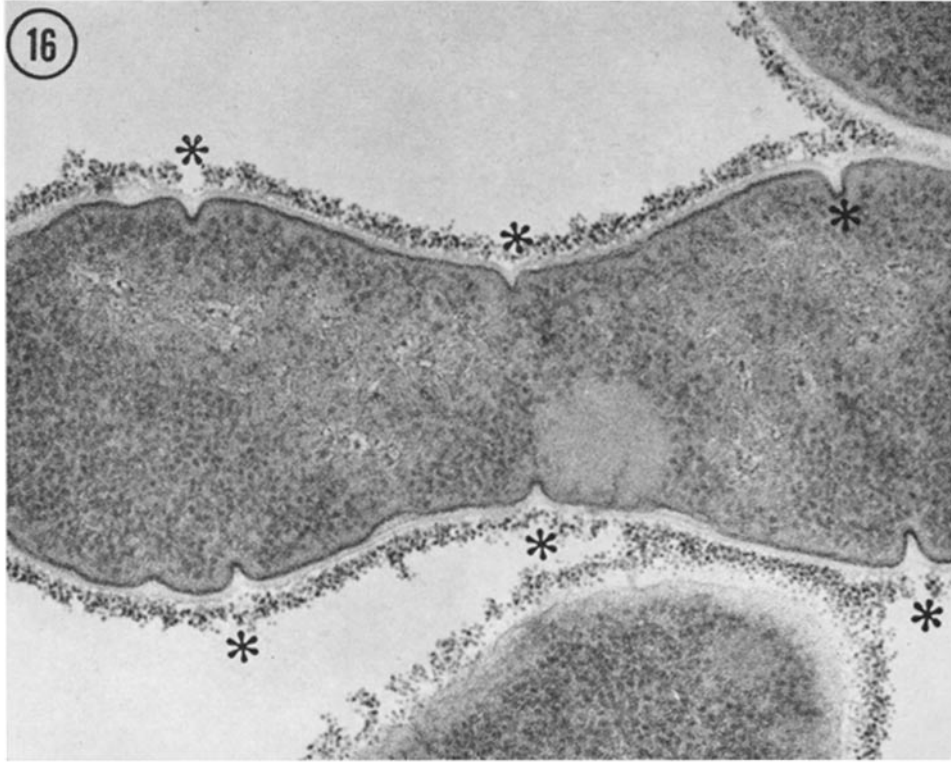


FIG. 16. Type 5 streptococci (strain T 5B) which have been exposed to ferritin-conjugated type 5 antibodies show heavy tagging by ferritin particles. Ferritin is largely absent on the surfaces in vicinities of beginning septum formation (*). Ferritin particles often assume a linear arrangement which mimics the form of surface fimbriae whose outlines are obscured. $\times 60,000$.

FIG. 17. Control, heterologous type 6 (strain S 43/100/15) streptococci have few ferritin particles on their exteriors after incubation in ferritin-conjugated type 5 antibodies. The ferritin particles (arrows) are located at the tips of the surface fimbriae. $\times 60,000$.

FIG. 18. Type 5 (strain T 5B) streptococci were exposed to ferritin-conjugated type 5 antibodies prior to trypsin treatment of the remainder of the culture. Sites of beginning septum formation (*) are tagged with fewer ferritin particles than the remainder of the heavily tagged cell wall surface. $\times 60,000$.



The organisms shown in Figs. 19–22 were exposed to trypsin and then reincubated in trypsin-free broth. Specimens were briefly fixed at the end of trypsin treatment (Fig. 19) and after 10 min (Figs. 20a & 20b), 30 min (Fig. 21), and 60 min (Fig. 22) reincubation. These specimens and the nontrypsinized, fixed control (Fig. 18) were exposed to ferritin conjugated antibodies under identical conditions after their primary brief fixation and washing.

FIG. 19. At the end of trypsin treatment, the type 5 streptococci do not tag with ferritin-conjugated type 5 antibody. The cell wall surfaces are devoid of fimbriae. $\times 60,000$.

FIGS. 20a & 20b. After 10 min reincubation in trypsin-free broth, type 5 streptococci have abundant ferritin particles on their surfaces near developing (Fig. 20a) and nearly completed (Fig. 20b) intercellular septa. Sparse ferritin tagging is present on most of the remainder of the cell wall surfaces. Heavy ferritin tagging is also seen in the crevices between daughter cells that have nearly completed assumption of spherical forms. $\times 60,000$.

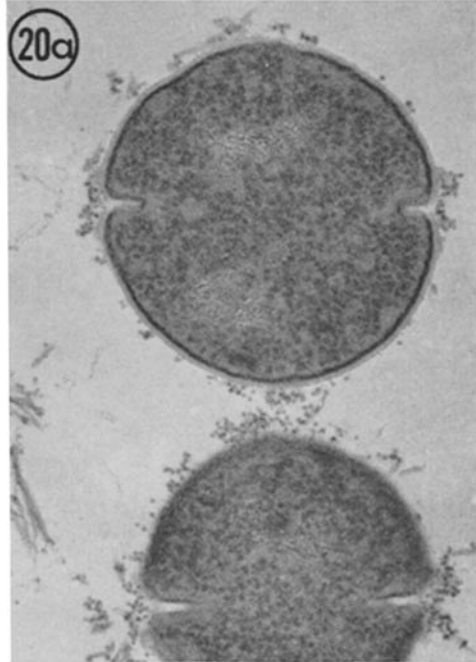
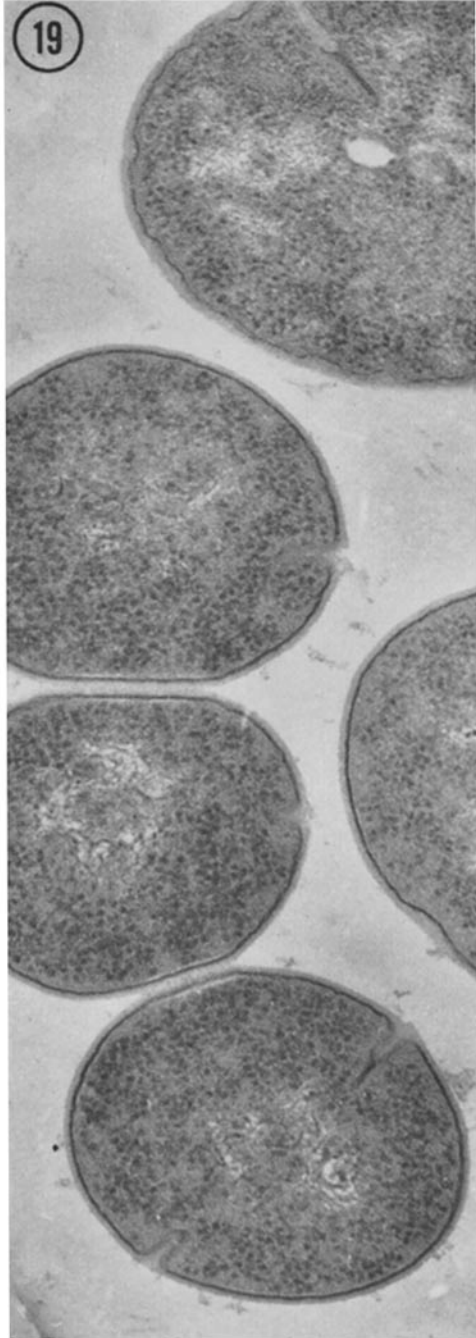


FIG. 21 After 30 min reincubation in trypsin-free broth, the streptococcal cell walls are tagged by ferritin particles in two density pattern. Abundant ferritin particles (a) are present on those parts of the cell wall that are probably newly synthesized. Sparse ferritin particles (s) coat the remaining "old" portions of the cell wall. The latter probably represent cell wall segments that were present at the end of trypsinization, i.e., they are not newly synthesized. Several cross septa that are beginning to form are covered by few or no ferritin particles (*). $\times 60,000$.

FIG. 22. After 60 min reincubation in trypsin-free broth, nearly all parts of the streptococci are covered by ferritin particles. The majority of the cell wall surfaces exhibit abundant ferritin particles (a). In a few areas ("old" segments of cell wall) ferritin particles are sparse (s). One beginning cross septum is not covered by ferritin particles (*).

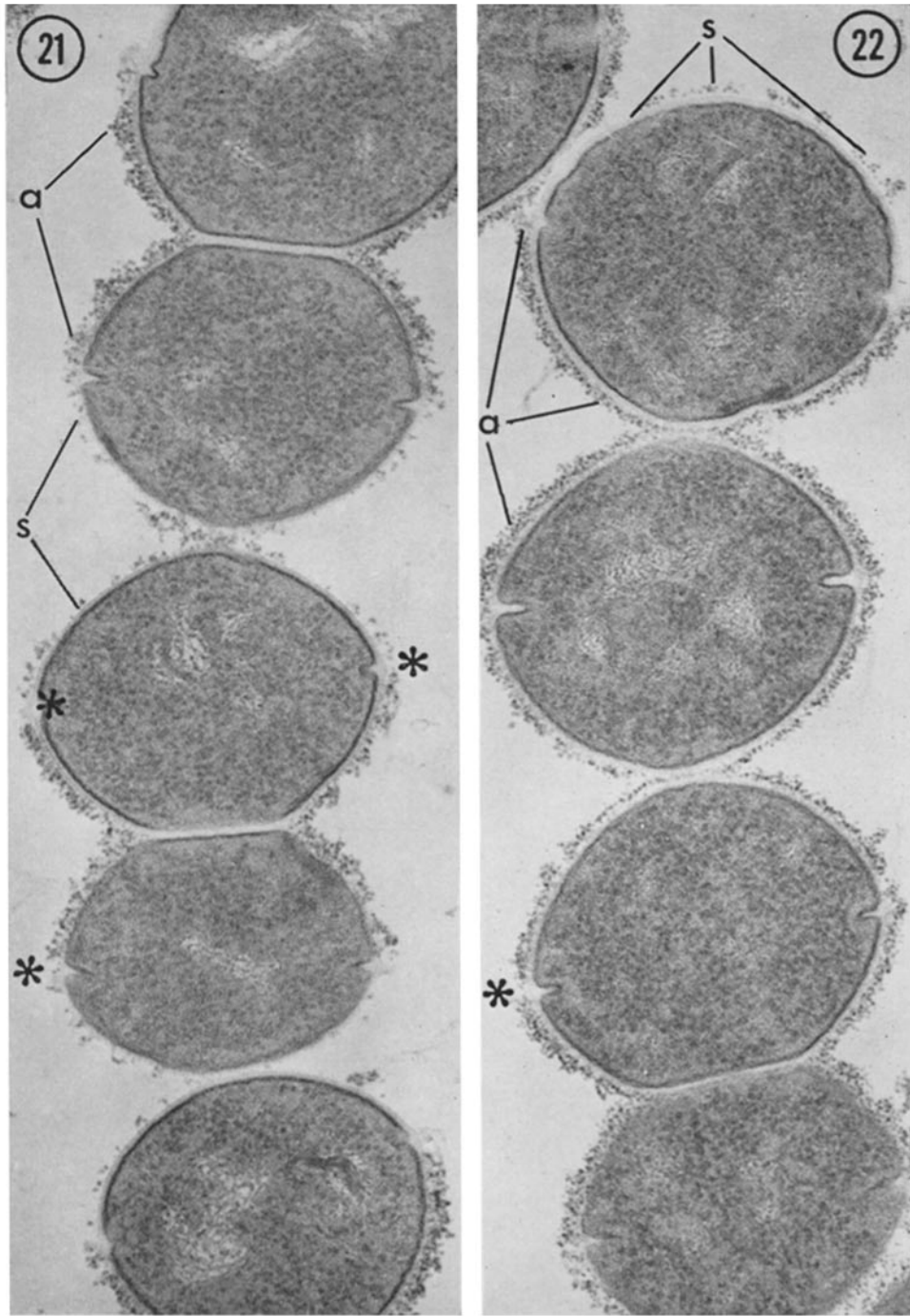


FIG. 23. Type 12 streptococci fixed prior to nitrous acid extraction exhibit numerous fimbriae on their surfaces (inset, $\times 60,000$). At higher magnification the compact laminae of the cell wall are easily seen. The outer compact lamina (between white and black arrows) is 140 A thick. $\times 250,000$.

FIG. 24. Type 12 streptococci fixed after nitrous acid extraction also exhibit fimbriae on their surfaces (inset, $\times 60,000$). The fimbriae are attached to the thinned portion of the cell wall (75–80 A, between white and black arrows) which is derived from the outer compact lamina of the untreated streptococcal cell wall. $\times 250,000$.

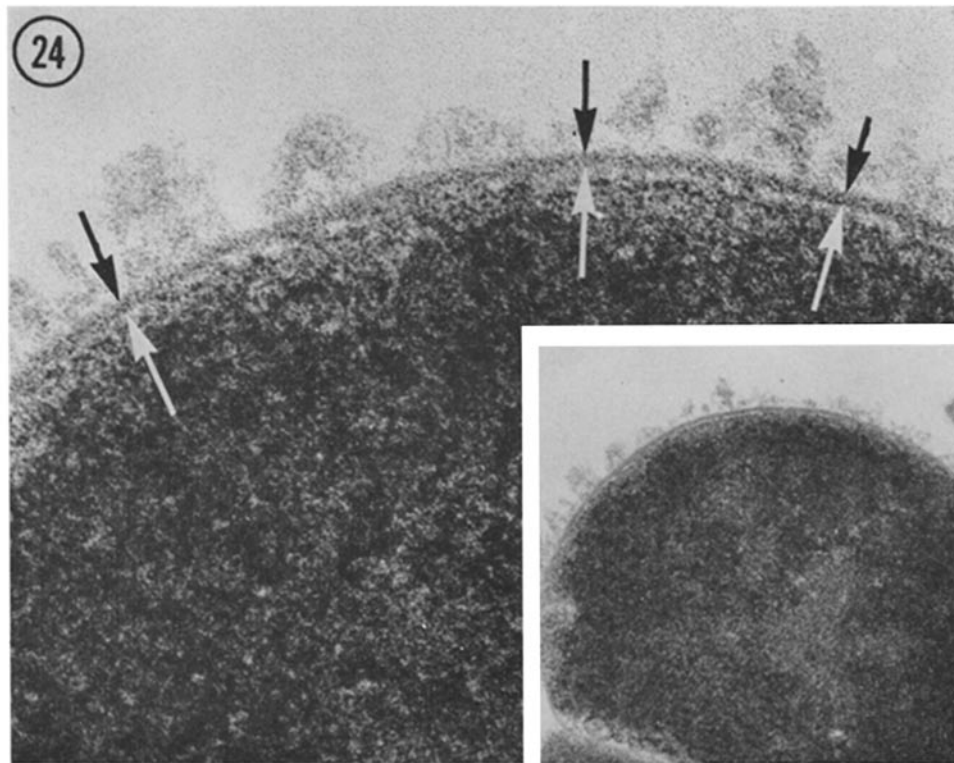
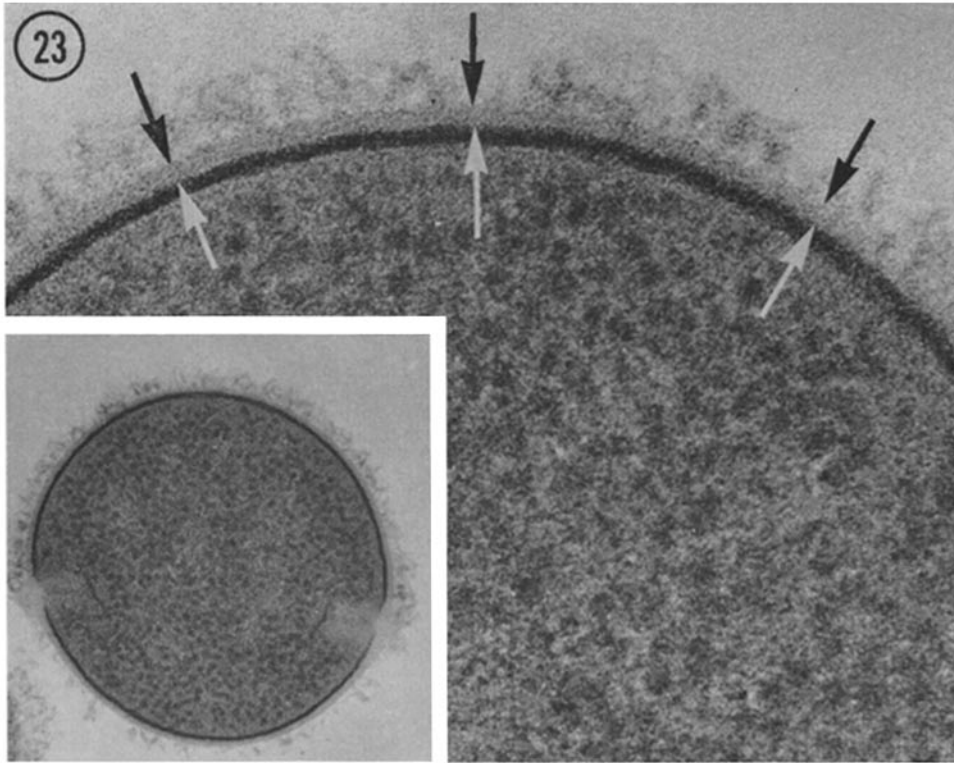


FIG. 25. Type 5 streptococci have abundant ferritin particles on their surfaces after exposure to ferritin-conjugated type 5 antibodies before nitrous acid extraction (inset, $\times 60,000$). At higher magnification, the ferritin particles can be seen to assume a linear distribution (arrows) similar to the orientation of surface fimbriae. The overall morphology and thickness of the compact laminae of the cell wall are similar to those of cells in Fig. 23. $\times 250,000$.

FIG. 26. Type 5 streptococci were extracted with nitrous acid and were then exposed to ferritin-conjugated type 5 antibodies. Ferritin particles are numerous on the surfaces of these extracted cells (inset, $\times 60,000$). Ferritin is often seen distributed in linear patterns (arrows) that radiate from the thinned residuum of the nitrous acid-extracted cell wall. This remaining portion of the cell wall is similar in thickness to that seen in Fig. 24. $\times 250,000$.

