INFECTION AND IMMUNITY, July 1983, p. 410–413
0019-9567/83/070410-04$02.00/0

Vol. 41, No. 1

NOTES

Ultrastructural Localization of Protein Antigens I/II and III in *Streptococcus mutans*

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Received 1 March 1983/Accepted 26 April 1983

Peroxidase-conjugated antibodies to antigens I/II and III were used to stain cells of *Streptococcus mutans* serotypes *a* through *g*. Under the electron microscope, serotypes that possessed these antigens showed deposits of peroxidase reaction products in association with the cell surface.

Protein antigens I/II and III of *Streptococcus mutans* were first identified in culture supernatants (10) and subsequently have been purified from both culture fluids and cells (8, 9). Antigen I/II is a protein of molecular weight 185,000 which has been identified in culture fluids and cells of *S. mutans* serotypes *a*, *c*, *d*, *e*, *f*, and *g* (9). It is partially susceptible to common proteases which remove the determinants composing antigen I and leave a resistant protein core of molecular weight 48,000 to 69,000 called antigen II (9). Antigen III is a protein which is resistant to most common proteases; it has a molecular weight of approximately 39,000 and is found in culture fluids and cells of *S. mutans* serotypes *b*, *c*, *e*, and *f* (8). The biological functions of these proteins have not been elucidated, but they are distinct from glucosyltransferase and dextran-binding proteins. However, antigen I/II, and its component parts, antigens I and II, can induce protective immunity against dental caries in monkeys (5). This property correlates with the ability of antisera to these proteins to opsonize *S. mutans* for phagocytosis by polymorphonuclear leukocytes (13). These observations suggest that antigen I/II is important in interactions with the host and its immune defense system. The present investigation was undertaken to determine precisely where the antigens are located, using electron microscopy and peroxidase-labeled antibodies.

S. mutans strains AHT (serotype *a*), BHT (*b*), MT-8148 (*c*), SL-1 (*d*), LM-7 (*e*), OMZ-175 (*f*), and 6715-DP (*g*) were kindly provided by Suzanne M. Michalek and were grown overnight in brain heart infusion broth.

Monospecific antisera to antigens I/II and III were raised in rabbits immunized with purified proteins, as described previously (8, 9). Immunoglobulin G (IgG) fractions of these sera and of normal rabbit serum were prepared by chromatography on DEAE-cellulose in 0.0175 M phosphate buffer (pH 6.5) followed by precipitation with 50% saturated ammonium sulfate. These IgG preparations (5 mg) were conjugated with horseradish peroxidase (type VI; Sigma Chemical Co.) (5 mg) by the method of Nakane and Kawaoi (6). Briefly, this consists of treating the peroxidase first with fluorodinitrobenzene to block free amino groups and then with periodate to oxidize carbohydrate moieties to aldehyde groups, which then may be coupled to IgG by forming Schiff bases with free amino groups.

Air-dried smears of washed *S. mutans* cells were treated with the peroxidase conjugates diluted to 50 μg/ml in phosphate-buffered saline (pH 7.4) for 30 min at room temperature and washed in saline for 30 min. The substrate, consisting of 30 mg of 3,3′-diaminobenzidine tetrahydrochloride dissolved in 100 ml of 0.05 M Tris-hydrochloride (pH 7.6)–0.005% (wt/vol) hydrogen peroxide, was applied for 30 min in the dark. The slides were rinsed in water, dried, and examined with an oil immersion objective.

Suspensions of *S. mutans*, grown overnight and washed as described above, were treated with the peroxidase conjugates at approximately 0.5 mg/ml for 30 min. The cells were washed three times with phosphate-buffered saline and fixed for 10 min in 2% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After a further five washes for 2 h in phosphate-buffered saline

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the cells were immersed in substrate (described above) for 10 min, washed three times in phosphate-buffered saline, and then treated with 2% (wt/vol) osmium tetroxide for 1 h. The cells were finally dehydrated and embedded in Spurr medium for sectioning. Thick sections were examined by light microscopy, and ultrathin sections were examined in a Philips TEM-300 electron microscope.

Brown peroxidase reaction products could be observed on positive slides by the unaided eye within a few minutes, and under the light microscope they were seen associated with the bacterial cells. By these criteria, it could be seen that antigen I/II was present in strains of serotypes *a*, *c*, *d*, *e*, *f*, and *g* and that antigen III was present in strains of serotypes *b*, *c*, *e*, and *f* (Table 1). No strain was stained by the control conjugate, even when this was applied undiluted and the substrate was allowed to react for 2 h. These results confirm those obtained previously by indirect immunofluorescence (8, 9) and show that peroxidase conjugation did not affect the specificity of the antibodies.

Sections of *S. mutans* stained with lead citrate and uranyl acetate were examined in the electron microscope first to comprehend the cellular structure (Fig. 1A). The cells appeared generally as diplococci in longitudinal section. An electron-dense line on the inner aspect of the cell wall probably represented the inner wall structure and cell membrane (4). The thick outer layer of the cell wall was of uniform low density. On some cells, the outer surface appeared rough, but no other evidence of a "fuzzy coat" or fimbriae was seen.

Immunoperoxidase-stained *S. mutans* cells were subsequently examined without heavy-metal staining, to detect the peroxidase reaction products more clearly. There was no evidence of reaction products in preparations of cells treated with the control conjugate (normal IgG) (Fig. 1B). The cells appeared to be of medium density, and the walls appeared to be of low density, but the osmiophilic cell membrane could be seen in places as a line of high density.

When stained with the anti-antigen I/II conjugate, cells of positive strains (e.g., serotypes *c* and *g*) revealed electron-dense reaction products localized on the outside of the cell wall (Fig. 1C and D). Cells of serotypes *c*, *e*, and *f* often displayed large globular masses of reaction products on the cell wall surface, and dense products in the outer layer of the cell wall, but those of serotypes *a*, *d*, and *g* appeared to contain less-abundant deposits. The cells of serotype *b*, which does not possess antigen I/II, did not reveal reaction products when stained for this antigen. Staining for antigen III revealed reaction products of similar appearance to those seen with antigen I/II, but only on cells of serotypes *b*, *c*, *e*, and *f* (Fig. 1E and F). With serotypes *c*, *e*, and *f*, abundant superficial deposits were seen, and in all positive strains, the cell wall appeared to be uniformly stained.

The antigens appeared to be distributed over the entire cell surface. In no strain examined did there appear to be any segmental distribution of staining for either antigen, such as in polar regions or in developing cross-walls or their associated membranes. Furthermore, when some cultures were examined in the logarithmic growth phase (as evidenced by longer chain formation and shorter cell length), the presence and distribution of reaction products were the same as in the stationary-phase cells.

Some cells contained electron-dense reticulate membranous structures, which could be quite large. Whether these were true mesosomes or artifacts induced by the treatment procedures is not clear. However, their density, and that of the inner wall-membrane zone, probably did not represent the presence of antigen, as they were not stained noticeably darker by the relevant specific antibody conjugates. Furthermore, it is unlikely that the conjugates could have penetrated the cell wall in intact cells.

These results confirm, at the electron microscopic level and with the immunoperoxidase technique, previous observations on the occurrence of antigens I/II and III in different serotypes of *S. mutans* (8, 9). Furthermore, antigens I/II and III were demonstrated to be present on the outer surface of the cell wall structure. Thus, it appears to be correct to regard these proteins as cell wall or surface antigens. They may form the protein fuzzy coat that has been reported by others (4). Microcapsules have been reported on cells of several strains of *S. mutans* (2). These were demonstrated by means of peroxidase-conjugated antisera, but the precise specificity

TABLE 1. Reactions of peroxidase-conjugated antibodies with *S. mutans* strains

Strain	Serotype	Reaction[a] with the following peroxidase conjugate:		
		Control IgG[b]	Anti-I/II[c]	Anti-III[c]
AHT	*a*	−	+	−
BHT	*b*	−	−	++
MT-8148	*c*	−	+++	+++
SL-1	*d*	−	++	−
LM-7	*e*	−	++	++
OMZ-175	*f*	−	+++	++
6715-DP	*g*	−	++	−

[a] −, No reaction; +, weak reaction; ++, moderate reaction; +++, strong reaction.
[b] Undiluted.
[c] Diluted 10-fold.

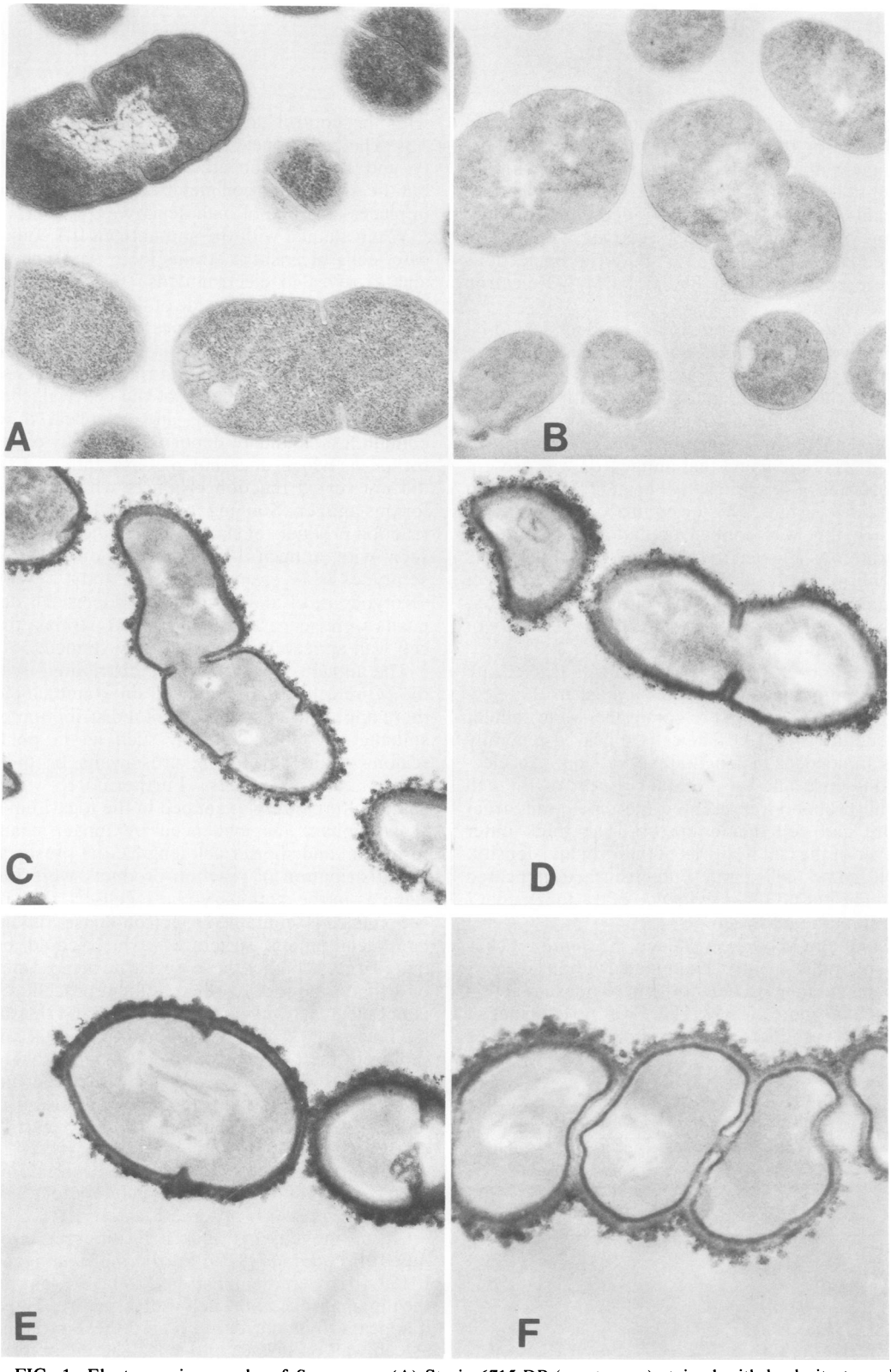

FIG. 1. Electron micrographs of *S. mutans*. (A) Strain 6715-DP (serotype *g*) stained with lead citrate and uranyl acetate. Magnification, ×40,000. (B) Strain MT-8148 (serotype *c*) stained with control conjugate. Magnification, ×40,000. (C) Strain MT-8148 stained with anti-antigen I/II conjugate. Magnification, ×33,000. (D) Strain 6715-DP stained with anti-antigen I/II conjugate. Magnification, ×55,000. (E) Strain MT-8148 stained with anti-antigen III conjugate. Magnification, ×42,000. (F) Strain LM-7 (serotype *e*) stained with anti-antigen III conjugate. Magnification, ×55,000.

of these sera was not examined. However, as our results appear qualitatively similar, it is possible that the microcapsular structures included antigens I/II and III. Craig et al. have shown that antibody to the serotype *a* polysaccharide reveals a dense microcapsule on the cell surface of strain AHT (1). As the polysaccharide was released into the culture medium in association with 5% protein, they speculated on the presence of protein in the microcapsule.

It appears that antigens I/II and III are present throughout the growth cycle, and their presence in the culture medium is believed to be due to the sloughing off of the extracellular globular masses seen in this study.

The molecular relationship of antigens I/II and III to other structural components of the cell wall is not known. However, the fact that at least some molecules of these antigens resist extraction of broken cell wall preparations in hot solutions of sodium dodecyl sulfate (11; M. W. Russell, unpublished data) implies covalent bonding with other components. Covalently bonded proteins in the cell walls of *S. mutans* have been reported by others (7).

Since our original description of protein antigens in *S. mutans* (10), other investigators have reported antigens that have similar characteristics (3, 11). Comparison of these materials by means of Ouchterlony immunodiffusion and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate has shown that antigens P1 and B are indistinguishable from antigen I/II of molecular weight 185,000 (9; H. Forester, N. Hunter, and K. W. Knox, J. Gen. Microbiol., in press; M. W. Russell, unpublished observations). Similarly, it has been found that antigen A is identical to antigen III of molecular weight 39,000 (8; M. W. Russell, unpublished data).

The presence of both of these antigens on the outer surface of *S. mutans* is relevant to the reports that they may induce protective immunity to dental caries (5, 12). It also explains how antisera to antigen I/II, although not antisera to antigen III, are able to opsonize *S. mutans* (13). The reasons for this discrepancy are not clear, but they could involve the way in which the two antigens are associated with the cell wall. The surface location of these antigens, however, is consistent with the notion that they may be important in interactions between *S. mutans* and its host. Of particular interest will be the possibility that antigens I/II and III may be involved in colonizing tooth surfaces, as well as in confrontations with the immune defense system of the host.

We thank Gerald Shockman and John Barrett for helpful discussions.

This study was partly supported by Public Health Service grant DE-02670 from the National Institutes of Health.

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