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Characterization of an Antibody Directed Against a Surface Component of Normal and Pleomorphic Cells of *Streptococcus sanguis*

ROSELYN J. EISENBERG* AND PAUL C. MONTGOMERY

Department of Microbiology and Center for Oral Health Research, University of Pennsylvania, School of Dental Medicine, Philadelphia, Pennsylvania 19174

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Whole cells of *Streptococcus sanguis* were utilized as an immunoadsorbent to purify large quantities of an antibody (S1) directed against a cell surface component. The S-1 antibody was isolated from antisera to normal (N) and pleomorphic (O) cells by a similar adsorption-elution procedure. The S-1 antibody isolated from antisera to N cells reacted in gel diffusion in identity with the S-1 antibody to O cells, indicating that the antigen which binds S-1 antibody (Ag-1) may not be radically altered when cells become pleomorphic. The S-1 antibodies directed against both N and O cells had restricted heterogeneity, indicating that for both types of cell Ag-1 may have a simple repeating structure. However, N cells were agglutinated to a greater extent by S-1 antibody than O cells. In addition the distribution of the bound S-1 antibody became altered as the cells became pleomorphic. Utilizing the technique of indirect immunofluorescence we observed that the S-1 antibody was distributed evenly on the surface of N cells. As the cells became pleomorphic, the antibody appeared to bind preferentially at the cell poles (capping). Later, as the cells became more grossly deformed, additional bands of immunofluorescence appeared to bisect the cells. Electron microscopic analysis indicated that the bound antibody was not associated with septal notches. The results suggest that the arrangement rather than the immunological properties of Ag-1 became altered as cells became pleomorphic.

Cells of *Streptococcus sanguis* undergo gross changes in morphology when cultured in the presence of oxygen (O cells) (4). Normal growth and morphology require that the organisms be grown in a nitrogen atmosphere (N cells). The changes in morphology include enlargement of the cells, distortion of the normal coccal shape, and formation of multiple incomplete septa. We also observed that macromolecular synthesis was unbalanced in O cells. Ribonucleic acid (RNA) and protein synthesis were inhibited while deoxyribonucleic acid (DNA) and glycogen synthesis were enhanced significantly (4). A preliminary analysis of the gross composition of the cell walls of N and O cells revealed that O cells contained a higher ratio of rhamnose/peptidoglycan than did N cells (B. Rosan, personal communication). These results taken together suggested that O cells suffered major disruptions in the control of macromolecular synthesis. Such disruptions especially in reactions concerned with polysaccharide synthesis might be the cause of the morphological changes that were observed to occur. Furthermore, these changes might be reflected by alterations of the cell surface structure of O cells.

Two kinds of alterations could be expected to lead to changes in cell surface structure. First, there might be quantitative changes in cell wall components; some components could be synthesized excessively while others might not be made at all. Another possibility is that new wall components are made. In *Bacillus subtilis*, a temperature-sensitive *rod*$^-$ mutant has been isolated which is deficient in teichoic acid but makes excessive amounts of peptidoglycan at the nonpermissive temperature (1). Another more subtle change might involve a rearrangement of cell wall components. In *Arthrobacter*, for example, it was shown that the transition from sphere to rod involves an alteration in the arrangement of peptidoglycan components rather than in overall composition (10, 11). The rod form of the organism appears to contain a more complex and rigid structure with additional side chains. The sphere form appears to be a relatively loose network with fewer side chains (11). Thus, studies of the arrangement

of cell wall components might be a more sensitive method of detecting cell surface changes than studies of cell wall composition. We therefore decided to study the effect of oxygen on the arrangement of one specific antigenic component of the cell surface. We reasoned that the utilization of an antibody directed against an immunodominant cell surface antigen would permit us to make morphological comparisons of N and O cells without having to purify the components involved in the alteration.

Several groups (2, 18, 24) have observed that when antiserum to a streptococcus is mixed with formalin-treated whole cells or cell walls of the organism, the cells act as an immunoadsorbent. The bound antibody is easily removed under acidic and nondenaturing conditions. In the case of *S. mutans*, the monoprecipitin antibody eluted in this fashion has been shown to be directed against the group carbohydrate (8).

In the present study, a similar absorption elution procedure was used except that the cells were untreated before adsorption and acetic acid was used as the eluant. Our results suggest that there was no detectable change in the structure of Ag-1 in pleomorphic cells. However, there appeared to be a major alteration in the amount and arrangement of Ag-1 on the O cell surface. The possible reasons for the alteration are discussed.

MATERIALS AND METHODS

Bacterial strain and culture technique. The biological properties of *S. sanguis* (strain G9B) used in these studies have been described previously (4). For rabbit immunizations, 200 ml of brain heart infusion broth (Difco) was inoculated with 10 ml of an 18-h seed culture of *S. sanguis* and incubated at 37 C in an atmosphere of 95% N_2 plus 5% CO_2 for N cells or in an atmosphere of 95% O_2 plus 5% CO_2 for O cells. Cell growth was followed as previously described (4). When the culture reached stationary phase (5 h), 2 ml of 37% formaldehyde was added and incubation was continued for an additional hour. The suspension was centrifuged at 10,000 × *g* for 15 min, and the cells were washed twice with sterile 0.15 M NaCl-0.1 M phosphate buffer (PBS), pH 7.2, resuspended in PBS to a cell concentration of 1×10^{10} cells/ml, and stored at 5 C.

For antibody purification experiments, 4-liter cultures were grown as previously described. The cells (6 g, wet weight) were washed three times with 0.15 M NaCl-0.05 M borate, pH 8.4 (BBS), and resuspended in this buffer to a cell concentration of 5×10^{10} cells/ml. Cell concentrations were determined by direct microscopic count as described previously (4).

To obtain cell-free extracts, the cells from 4 liters were suspended in 10 ml of BBS, chilled to 0 C in an ice water bath, and sonically treated in a Lab-Line Sonifier at a setting of 40 to 60 W for 10 to 15 min. This procedure resulted in 80 to 100% cell breakage by microscopic examination. The suspension was centrifuged at 8000 × *g* for 15 min, and the supernatant fluid was collected. Crude extracts usually contained 40 to 50 mg of protein/ml as determined by the method of Lowry et al. (14).

Immunization procedure. New Zealand white rabbits were injected intravenously three times a week for 4 weeks with the following concentrations of formalin-treated N or O cells: week 1, 1×10^8 cells; week 2, 5×10^8 cells; week 3, 1×10^9 cells; and week 4, 5×10^9 cells. After a 4-week rest, the rabbits were boosted with another series of injections. Bleedings were taken at weekly intervals by cardiac puncture during the immunization schedule. Sera obtained from each monthly immunization were pooled for each individual rabbit. Prior to antibody purification, the serum pools were heated at 56 C for 30 min to inactivate complement.

Antibody purification. Ten milliliters of *S. sanguis* cells in BBS (5×10^{10} cells/ml) was mixed with 10 ml of crude heat-treated antiserum or with normal rabbit serum (NRS) as a control. The mixtures were incubated for 15 min at 37 C with gentle stirring and then centrifuged at 20,000 × *g* for 20 min. The unadsorbed serum was decanted, and the cells were washed three times with 15 ml of cold BBS. After the washing, little or no 280-nm absorbing material was detectable in the supernatant. The cell pellet was suspended in 15 ml of 0.01 M acetic acid, incubated for 10 min at 5 C, and then centrifuged at 20,000 × *g* for 20 min. The eluate was decanted and saved. This procedure was repeated successively on the cell pellet with 15 ml of 0.1, 1, and 4.4 M acetic acid. The acetic acid eluates were dialyzed exhaustively against 0.01 M phosphate buffer, pH 7.8, and concentrated four- to fivefold by ultrafiltration with a Diaflo PM 30 ultrafiltration membrane (Amicon Corp.). The concentrated samples were passed through a 0.45-μm membrane filter (Millipore) to remove residual bacteria and stored at −10 C. Anti-dinitrophenyl (DNP) antibodies were isolated from the sera of a rabbit immunized with dinitrophenylated bovine gamma globulin employing a DNP-Sepharose immunoadsorbent column (17). The 1 and 4.4 M acetic acid eluates were pooled and handled in a manner identical to that utilized for the S-1 antibodies. The anti-DNP antibodies were also immunoglobulin G (IgG).

Immunoelectrophoresis and agar diffusion. The microtechnique of Scheidegger was employed for all immunoelectrophoretic analyses (23). Specific antisera (Miles Laboratories) directed against rabbit serum proteins immunoglobulins G, A, and M were additionally absorbed with purified light chains (1 to 5 mg) until rendered specific for γ, α, and μ heavy chains. These antisera (2 to 4 mg of antibody/ml) were placed in the troughs after electrophoresis. The slides were developed overnight at room temperature and photographed. Agar diffusion was carried out on glass plates by the method of Ouchterlony (19). The IgG concentrations of normal and immune sera were determined by the method of Mancini et al. (15), using specific goat anti-rabbit IgG antiserum.

Isoelectric focusing. Aliquots (100 μl) of each purified antibody (250 to 270 μg) were analyzed by isoelectric focusing with Ampholine carrier ampho-

lytes (pH range 3.5 to 10, LKB-Produketer, AB, Stockholm) in thin layers of 5% polyacrylamide gels (17, 26). The focusing (final potential, 500 V) was continued for 18 h at 4 C. After completion of focusing, pH measurements were made and the antibody components were precipitated by soaking the gel in 10% trichloroacetic acid for 2 h. Gels were then stained with 0.1% Coomassie brilliant blue (in ethanol-acetic acid-water; 9:2:9, vol/vol/vol) for 2 h and destained with a solution of ethanol-acetic acid-water (6:1:13, vol/vol/vol) until residual background dye was completely removed.

Agglutination. A modification of the method described by Vedros and Hill (24) was employed. Cultures of *S. sanguis*, inoculated as previously described, were incubated for 3 h (midlog phase). At that time, 1 ml of a 5% solution of 2,3,5-triphenyl-2H-tetrazolium chloride was added per 30 ml of culture. Incubation was continued for an additional 2 h, and the cells (colored bright red) were harvested, washed with BBS, and suspended in BBS (2×10^9 cells/ml). The suspension could be stored at 4 C for a week with no visible effect on agglutination titers. Agglutination experiments were carried out in microtiter plates (Cooke Engineering Co., Alexandria, Va.) using BBS as diluent and 0.025-ml amounts of sera and cells. The plates were then centrifuged in an International centrifuge at $1,000 \times g$ for 15 min. The titers are expressed as the greatest dilution of serum or S-1 antibody that gave agglutination.

Fluorescent-antibody technique. The method of Holbrow and Johnson (7) was employed to prepare fluorescein-isothiocyanate (FITC)-labeled goat anti-rabbit IgG. The amount of FITC in the conjugate was calculated to be 70 μg/ml, and the ratio of FITC to protein was calculated to be 7.2. Using whole cells of *S. sanguis* G9B, the optimal dilution of FITC for use in fluorescent staining was determined to be 1:40.

Immunofluorescent labeling of cells was performed on cultures of N and O cells as follows. At 1-h intervals, culture samples were removed, washed with BBS buffer, and resuspended in BBS (diluted 100-fold) to a concentration of 1×10^8 cells/ml. Two samples were placed on each slide, allowed to air dry and then were heat fixed. On each slide, 0.1 ml of antibody S-1 from rabbit E5 (10 μg/ml in BBS) was added to one smear and normal rabbit IgG (10 μg/ml in BBS) was added to the other smear as a control. The slides were incubated in a moist chamber for 30 min at 25 C and then washed twice with BBS and once with distilled water. The slides were flooded with FITC-labeled goat anti-rabbit IgG, incubated in a moist chamber for 30 min at 25 C, and washed three times with PBS and once with water. A drop of glycerol-saline (9:1) was added to each smear and a cover slip was placed on the slide. Slides were examined in a Leitz microscope under oil immersion for fluorescence. The film used was high-speed Ektachrome (Kodak, ASA 160).

Electron microscopy. A modification of the method of Lai et al. (12) was utilized. Fifty-milliliter cultures of N or O cells were inoculated as previously described and incubated at 37 C for 5 h. The cells were harvested by centrifugation and then washed twice with PBS. The cell pellets were suspended in 1 ml of homologous S-1 antibody (5 to 7 mg/ml) and incubated 30 min at room temperature. The suspensions were centrifuged and washed twice in PBS, and the resulting pellets were sectioned, stained, and viewed in the electron microscope (12).

Chemical procedures. Samples for protein and RNA determinations were first precipitated with 5% trichloroacetic acid and heated at 90 C for 15 min. The supernatant was collected, and the precipitate was resuspended in 5% trichloroacetic acid and reheated at 90 C for 15 min. The supernatant was combined with the first trichloroacetic acid supernatant and assayed for RNA by the orcinol method (6) with yeast RNA (Miles) as the standard. The pellet was dissolved in 0.1 N NaOH and assayed for protein by the method of Lowry (14) with crystalline bovine serum albumin (Miles) as the standard.

RESULTS

Elution of S-1 antibody from N and O cells of S. sanguis. N and O cells of *S. sanguis* suspended in BBS buffer were each mixed with homologous antiserum according to the procedure in Materials and Methods. As controls, equal volumes of N and O cells were mixed with NRS. The material eluted from the cells with acetic acid was dialyzed and assayed for protein and RNA. The results of a typical experiment are shown in Table 1. When N cells were mixed with homologous antiserum, the greatest amount of protein was always eluted at an acetic acid concentration of 1 M. In contrast, when O cells were mixed with homologous antiserum, the greatest amount of protein was always eluted at an acetic acid concentration of 0.1 M. This suggests that the eluted protein might have been somewhat less tenaciously bound to O cells than to N cells or was more soluble under acidic conditions when bound to O cells. When cells were treated with NRS, almost no protein was eluted with acetic acid. A very small amount of RNA was found in all of the eluates. The highest concentration of RNA was found in each of the 4.4 M fractions, indicating that there might have been some cell disruption at this acetic acid concentration. Microscopically the N and O cells appeared to remain intact throughout the elution procedure. However, both kinds of cells became gram negative after treatment with 4.4 M acetic acid.

The material eluted at each acetic acid concentration from immune serum and NRS-treated cells was dialyzed, concentrated 2- to 10-fold, and reacted in gel diffusion against crude N or O cell extracts of *S. sanguis*. The results obtained for N cells are shown in Fig. 1. Similar results were obtained with the material eluted from O cells. A single precipitin band (Fig. 1A) was found when material eluted with 0.1 and 1.0 M acetic acid was reacted against

crude cell extracts. No precipitating antibody was found in the 0.01 or 4.4 M eluates nor in material eluted from NRS-treated cells (Fig. 1B). The antibody-containing eluates (0.1 and 1.0 M fractions) did not react in gel diffusion with crude antisera, indicating that these antibody fractions were not significantly contaminated with cellular antigens.

TABLE 1. *Elution of antibody S-1 from O and N cells of S. sanguis*

Sample	Acetic acid concn (M)	Protein eluted[a] (mg/15 ml)	RNA eluted[b] (mg/15 ml)
N cells + antiserum to N cells[c]	0.01	2.1	0.020
	0.1	11.7	0.033
	1.0	21.8	0.058
	4.4	7.9	0.123
N cells + NRS	0.01	0.0	0.024
	0.1	0.0	0.059
	1.0	0.38	0.096
	4.4	0.27	0.132
O cells + antiserum to O cells[d]	0.01	3.14	0.046
	0.1	26.4	0.011
	1.0	17.6	0.043
	4.4	16.1	0.077
O cells + NRS	0.01	0.0	0.025
	0.1	0.10	0.023
	1.0	0.50	0.047
	4.4	0.44	0.065

[a] Determined by the method of Lowry et al. (14).

[b] Determined by the orcinol method (6).

[c] Antiserum (10 ml) from rabbit E5 injected with *S. sanguis* N cells for two monthly courses of immunization.

[d] Antiserum (10 ml) from rabbit E2 injected with *S. sanguis* O cells for two monthly courses of immunization.

The 0.1 and 1.0 M eluates were pooled, dialyzed, and concentrated. The pooled S-1 antibody eluted from N cells (S-1-N) showed a band of identity in gel diffusion with the S-1 antibody eluted from O cells (S-1-O) using crude cell extracts from O cells (Fig. 2A) or N cells (Fig. 2B).

This adsorption-elution procedure was repeated with four other rabbits immunized against *S. sanguis* N cells and four rabbits immunized against *S. sanguis* O cells with essentially similar results; i.e., large amounts of the same monoprecipitin antibody were eluted from cells with acetic acid.

Characteristics of the S-1 antibody eluted from S. sanguis. When the purified S-1 antibodies were subjected to immunoelectrophoresis, they migrated to the position characteristic of IgG (Fig. 3b). In addition all S-1 antibodies reacted with specific anti-γ chain antisera and not with anti-α or anti-μ antisera in gel diffusion. The amount of IgG in the S-1 antibody preparations was quantitated by the Mancini technique (15). Table 2 shows that 88% of the protein in the S-1-N antibody preparation could be accounted for as IgG. For the S-1-O antibody 73% of the protein was IgG. Additional experiments indicated that the S-1 antibody comprised from 5 to 20% of the total IgG in unfractionated antiserum.

Isoelectric focusing was employed to compare the molecular heterogeneity of the S-1-O and S-1-N antibodies (Fig. 4). Track a shows the spectrum of the S-1-O antibody obtained from the serum of rabbit E2. Track b shows the spectrum of the S-1-N antibody obtained from the serum of rabbit E5. Track c shows the

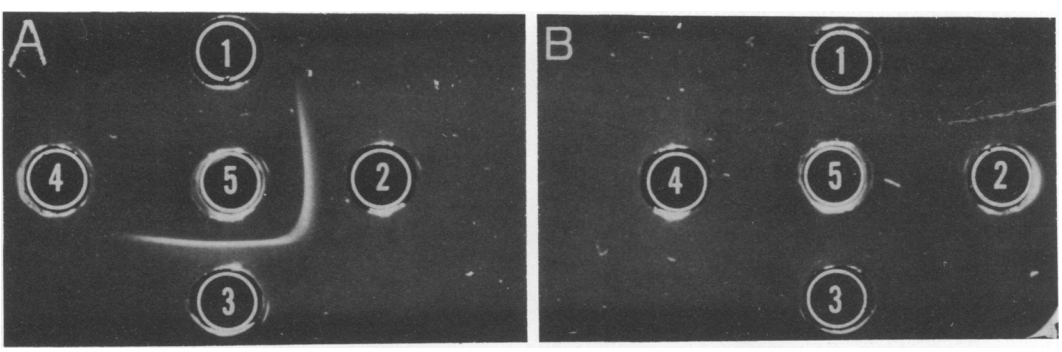

FIG. 1. *Gel diffusion analysis of material eluted from S. sanguis N cells by acetic acid. (A) Peripheral wells containing concentrated acetic acid eluates obtained by adsorption of rabbit E5 antiserum to S. sanguis cells; well 1, 0.01 M acetic acid eluate (1.4 mg/ml); well 2, 0.1 M acetic acid eluate (1.56 mg/ml); well 3, 1.0 M acetic acid eluate (2.9 mg/ml); and well 4, 4.4 M acetic acid eluate (2.08 mg/ml); well 5, crude S. sanguis N cell extract, 40 mg/ml of protein. No antibody was found in 0.01 and 4.4 M eluates or in material eluted from NRS-treated cells (B).*

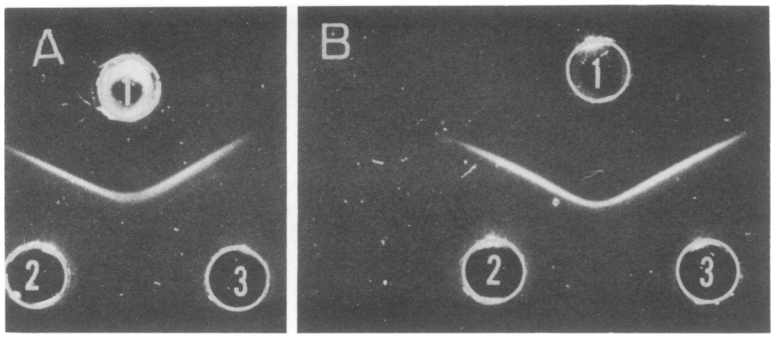

FIG. 2. *Gel diffusion analysis of S-1 antibodies isolated from antisera to O cells and N cells of S. sanguis. (A) Well 1, crude S. sanguis O cell extract (46 mg/ml of protein); well 2, S-1 antibody (1.78 mg/ml of protein) isolated by adsorption of serum from rabbit E2 to S. sanguis O cells and elution with acetic acid; and well 3, S-1 antibody (7.3 mg/ml of protein) isolated by adsorption of serum from rabbit E5 to S. sanguis N cells and elution with acetic acid. (B) Well 1, crude S. sanguis N cell extract (40 mg/ml of protein); well 2, S-2 antibody isolated from serum of rabbit E2 (1.78 mg/ml of protein); and well 3, S-1 antibody isolated from serum of rabbit E5 (7.3 mg/ml of protein).*

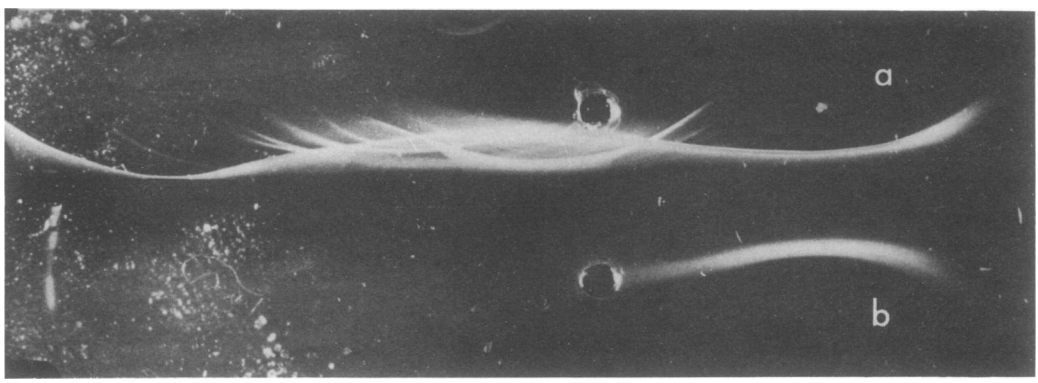

FIG. 3. *Immunoelectrophoretic patterns of crude antiserum to S. sanguis and antibody S-1. Well a, E5 antiserum. Well b, S-1 antibody (7.3 mg/ml) isolated by adsorption of serum from rabbit E5 to S. sanguis N cells and elution with acetic acid. The troughs contained goat antiserum directed against rabbit serum proteins.*

spectrum of purified anti-DNP antibodies obtained from the serum of a rabbit immunized with DNP-bovine gamma globulin. This latter spectrum typifies the antibody heterogeneity usually seen when rabbits are immunized with complex antigenic determinants (17) and is shown for comparative purposes. It is interesting to note the restriction in molecular heterogeneity of both S-1 antibodies (tracks a and b) compared to the anti-DNP antibody spectrum (track c). This pattern of restriction was maintained after repeated immunization in a total of four rabbits immunized against N cells and three rabbits immunized against O cells. In three other rabbits (one immunized against N cells and two immunized against O cells), the spectra were restricted in sera obtained after one monthly course of immunization but increased in complexity after repeated immunization with the appearance of additional cathodal bands. The spectral restriction suggested that the immunogen of N and O cells might be a simple repeating structure (16, 21, 27). Furthermore, although the S-1-O antibody might have become altered as the cells became pleomorphic, the alteration probably did not increase the complexity of the antigen since the S-1-O antibody from several rabbits consistently exhibited restricted heterogeneity.

If the surface of N and O cells is different, it might be expected that the S-1 antibody would have an altered ability to agglutinate O cells. We therefore assayed the agglutination of N and O cells by crude antiserum and S-1 antibody. The results (Table 3) indicate that N cells are more effectively agglutinated than O cells. The agglutination titer per milligram of IgG was twofold greater for N cells than for O cells. It should be noted that the same concentrations of O and N cells were used in these experiments. However, O cells are two- to threefold larger than N cells (4). Thus the greater amount of O

cell surface available to bind antibody might cause the agglutination titers to be lower due to a dilution effect. However, this explanation assumes that O cells have two to three times as many antibody binding sites as N cells.

It may be argued that the S-1-O antibody does not bind as well to cells as the S-1-N antibody. To answer this question, we mixed cells with the heterologous S-1 antibody. The results in Table 3 indicate that the heterologous antibody (S-1-N) has the same ability to agglutinate O cells as the homologous (S-1-O) antibody. Likewise, the heterologous (S-1-O) antibody has the same ability to agglutinate N cells as the homologous (S-1-N) antibody. These experiments suggest that the differences in agglutination properties of N and O cells are due to differences in the cells, rather than in the antibody.

Visualization of the binding of S-1 antibody to N and O cells of S. sanguis. (i) Visualization by dark-field fluorescence microscopy. To determine whether differences in agglutina-

TABLE 2. *Protein and IgG content of crude antisera and S-1 antibodies to N and O cells of S. sanguis*

Serum prepn	Protein[a] (mg/ml)	IgG[b] (mg/ml)	IgG (% of protein)[c]
Antiserum to N cells			
Unfractionated	77	12.7	16
S-1-N[d]	7.3	6.4	88
Antiserum to O cells			
Unfractionated	64	10.8	17
S-1-O[e]	1.78	1.3	73

[a] Determined by the method of Lowry et al. (14).

[b] Determined by the method of Mancini (15) employing goat anti-rabbit IgG antiserum.

[c] $\frac{\text{mg of IgG/ml}}{\text{mg of protein/ml}} \times 100.$

[d] The 0.1 and 1.0 M fractions eluted from N cells (Table 1) were pooled, dialyzed against 0.01 M phosphate buffer, pH 7.8, and concentrated.

[e] The 0.1 and 1.0 M fractions eluted from O cells (Table 1) were pooled, dialyzed against 0.01 M phosphate buffer, pH 7.8, and concentrated.

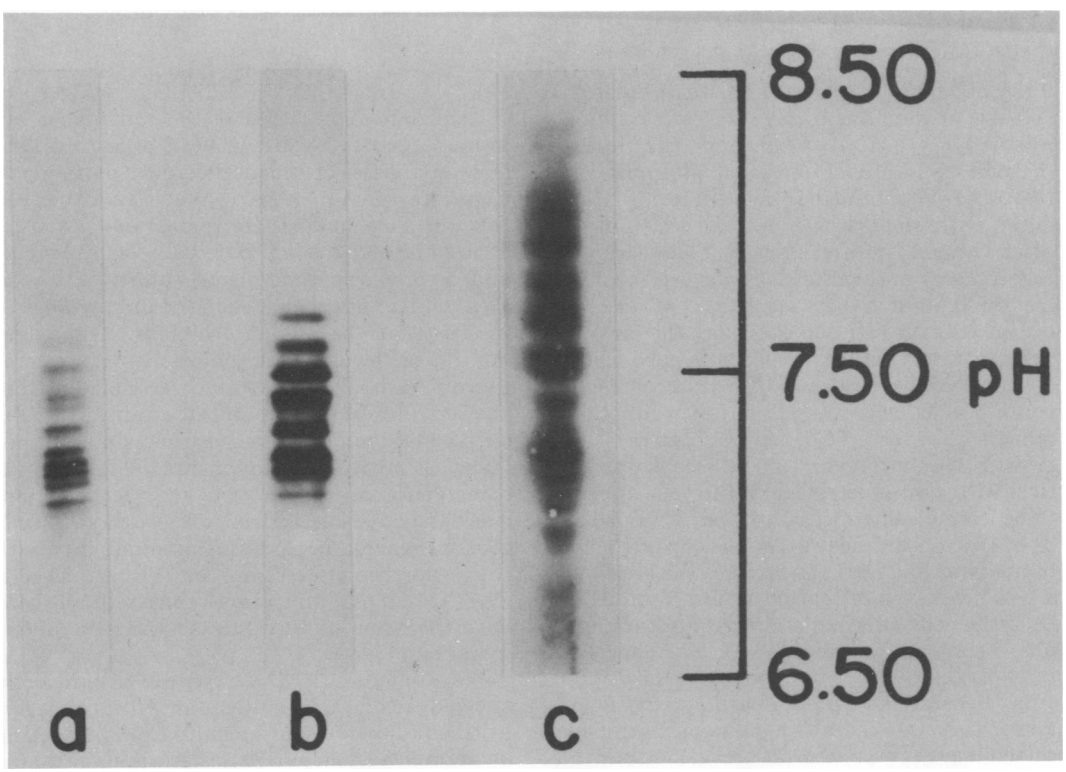

FIG. 4. *Isoelectric spectra of S-1 antibodies and anti-DNP antibody. Sera were subjected to isoelectric focusing in 5% polyacrylamide gels (pH range 3.5 to 10) and stained with Coomassie brilliant blue (for details see Materials and Methods). Track a: S-1 antibody (250 μg) isolated by adsorption of serum from rabbit E2 to S. sanguis O cells and elution with acetic acid. Track b: S-1 antibody (270 μg) isolated by adsorption of serum from rabbit E5 to S. sanguis N cells and elution with acetic acid. Track c: purified anti-DNP antibody (250 μg) obtained from serum of a rabbit immunized with DNP-bovine gamma globulin. Approximate pH values are shown.*

TABLE 3. *Agglutination of N and O cells of S. sanguis by crude antisera and S-1 antibodies*

Serum prepn	Cells	IgG (mg/ml)[a]	Agglutination titer[b]	Titer/mg of IgG
Homologous				
Crude antiserum to O cells[c]	O	11.7	512	44
Ab S-1-O[d]	O	1.8	64	36
Crude antiserum to N cells[e]	N	12.7	1024	81
Ab S-1-N[f]	N	6.4	512	80
Heterologous				
Ab S-1-O	N	1.8	128	71
Ab S-1-N	O	6.4	256	40

[a] Determination by the method of Mancini et al. (15).

[b] Determined according to method described in Materials and Methods with 2×10^9 cells/ml.

[c] Antiserum obtained after injecting rabbit E2 with O cells for two monthly courses of immunization.

[d] The 0.1 and 1.0 M fractions eluted from O cells (Table 1) were pooled, dialyzed, and concentrated.

[e] Antiserum obtained after injecting rabbit E5 with N cells after two monthly courses of immunization.

[f] The 0.1 and 1.0 M fractions eluted from N cells (Table 1) were pooled, dialyzed, and concentrated.

bility of N and O cells were due to differences in cell size or were due to differences in binding of antibody, we first employed the indirect method of immunofluorescent staining. Antibody S-1-N obtained from rabbit E5 was reacted with smears of N and O cells prepared after various times of growth. Fluorescein-conjugated goat anti-rabbit IgG was used to visualize the bound S-1-N antibody. At all times during the growth of N cells, the antibody appeared to encircle the cells evenly and the cells looked like those in Fig. 5-1. In contrast the coating of O cells was altered as a function of growth time (Fig. 5-1 to 5-5). After 1 h of growth, the morphology of these cells was identical with that of N cells and the cells appeared to be evenly coated with antibody (Fig. 5-1). At 2 h (Fig. 5-2), the poles of the cells were fluorescent but gaps appeared in the area where a new cross wall of septum would form. At this time the cells still had a normal appearance by phase contrast microscopy (4). The concentration of fluorescence at the poles or capping effect became more pronounced at 3 h (Fig. 5-3) and at this time there were obvious morphological changes. By 4 and 5 h (Fig. 5-4 and 5-5) additional bands of fluorescence appeared to bisect many of the cells. Occasionally these bands had the appearance of an X. Moreover, increasing the S-1 antibody concentration 10-fold did not change the patterns of binding seen in Fig. 5. Because of the lack of resolution of this technique we could not determine whether these bands were concentrated in regions of septation. However, these studies showed that as *S. sanguis* became pleomorphic, there was a continuous alteration of the cell surface which affected the binding of S-1 antibody.

(ii) Visualization by electron microscopy. To localize the bound S-1 antibody more precisely, we employed the technique of Lai et al. (12) (Fig. 6). We found that N cells were indeed coated evenly with S-1 antibody on all outside surfaces (Fig. 6a). The two elongated cells shown in Fig. 6b are representative of the two patterns of antibody binding seen with O cells. One cell, not fully elongated or grossly pleomorphic, had S-1 antibody concentrated at the poles of the cell. No antibody was bound to the cell in the septal region. The other cell, elongated and pleomorphic, had antibody localized at the polar regions and also had patches of antibody along the peripheral walls in areas which did not correspond to the septal notches. Thus it appears that the bands of antibody seen by immunofluorescence probably do not correspond in location to areas of septation.

DISCUSSION

Utilizing whole cells of *S. sanguis* as an immunosorbent, we have been able to isolate large quantities of a monoprecipitin antibody to a surface antigen (Ag-1). We have employed this antibody to compare the surface structure of normal and pleomorphic cells of *S. sanguis* and have shown that pleomorphism is associated with a radical change in the immunological properties of the cell surface. This change does not appear to involve a major chemical alteration in Ag-1. The S-1 antibodies isolated from the sera of rabbits immunized against N and O cells appear to be directed against similar if not identical antigenic components. Several kinds of evidence argue for such an interpretation. The antibody isolated from antiserum to N cells (S-1-N) reacted in gel diffusion in identity with the antibody isolated from antiserum to O cells (S-1-N). This finding argues that both antigens have the same or very similar antigenic determinants.

We also found that both types of S-1 antibody showed restricted molecular heterogeneity. Antibody heterogeneity results from presenting an animal with an antigenic determinant that is structurally complex (22). Reducing the complexity of the determinant generally reduces antibody heterogeneity (16, 22, 27). For example, in group A, A variant, and C streptococci (3, 9), as well as in type III and type VIII pneumococci (5, 20, 21), there are surface compo-

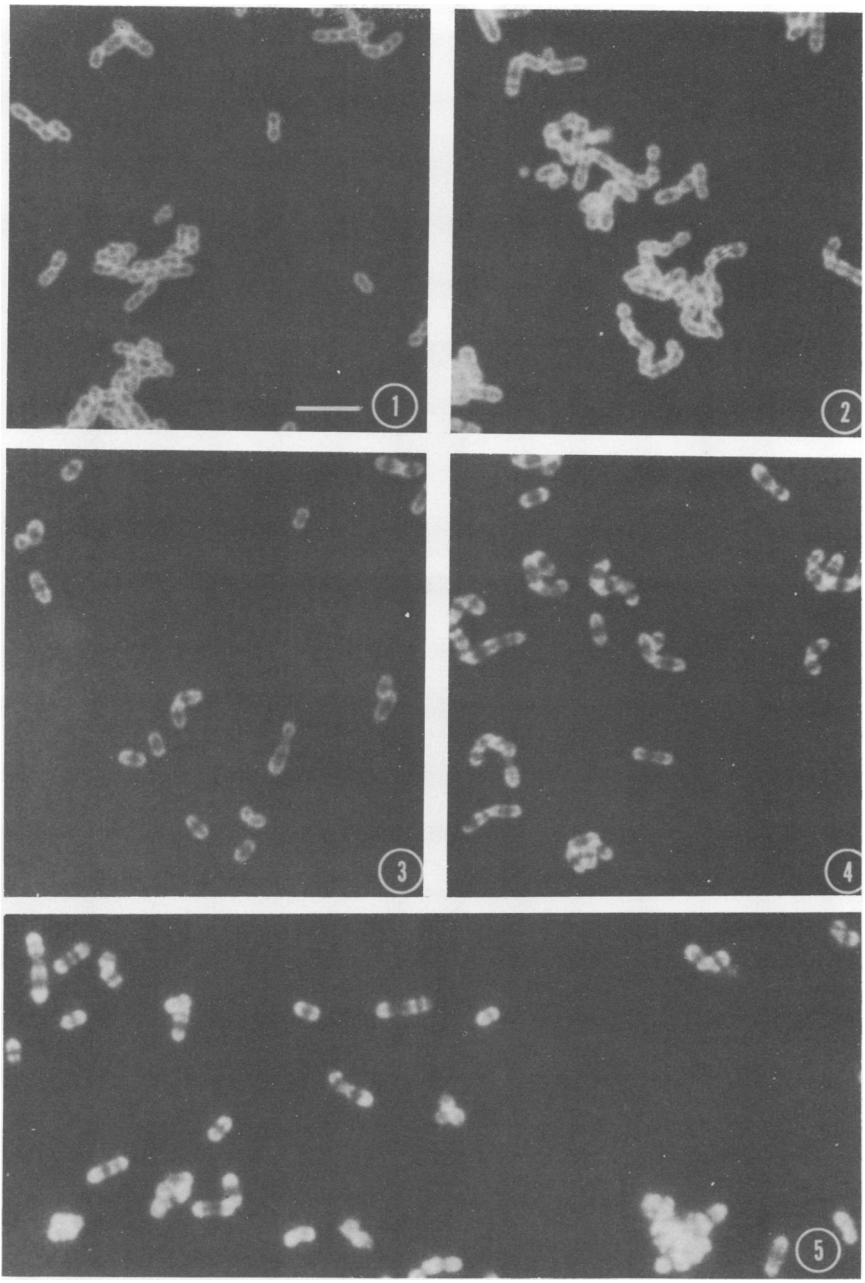

FIG. 5. *Fluorescent micrograph of O cells of S. sanguis reacted with S-1 antibody after different periods of growth. At the hours indicated by the circled numbers, cell samples were reacted with S-1 antibody (10 μg/ml) isolated by adsorption of serum from rabbit E2 to S. sanguis O cells and elution with acetic acid. The bound antibody was visualized by the indirect immunofluorescent technique (for details, see Materials and Methods). Marker bar, 5 μm.*

nents of either the wall or the capsule which consist chemically of simple repeating structures. In all of these systems, at some point during immunization, the antibody response has been shown to exhibit restricted heterogeneity. It is impossible to assess the extent to which this restricted response is influenced by genetic factors peculiar to each immunized animal (2). Nevertheless, the observation that the S-1-O and S-1-N antibodies obtained from different

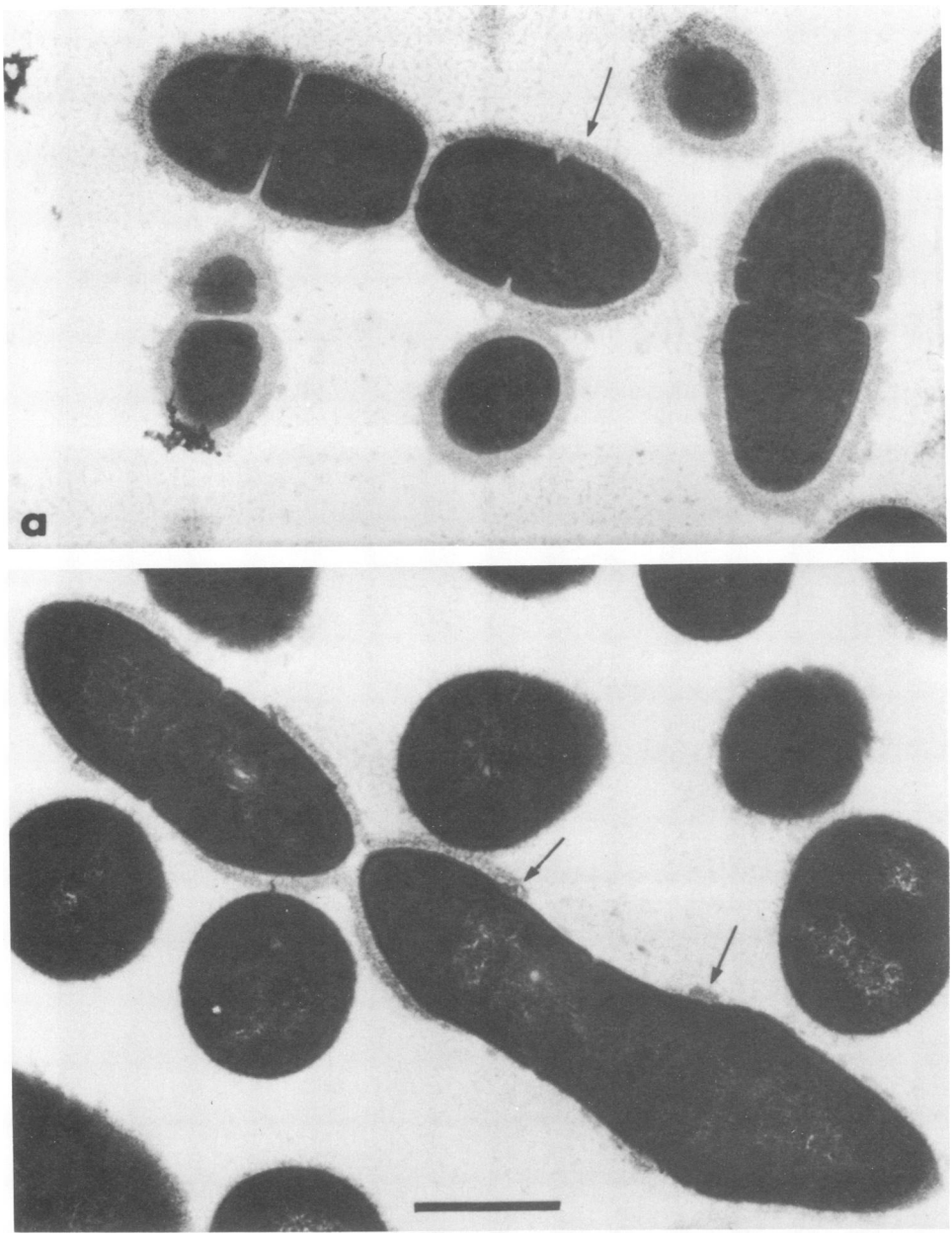

FIG. 6. *Electron micrograph of thin sections of S. sanguis N and O cells. (a) N cells incubated with S-1 antibody (1.78 mg/ml of protein) isolated by adsorption of serum from rabbit E2 to S. sanguis O cells and elution with acetic acid. (b) O cells incubated with S-1 antibody (1.78 mg/ml of protein) isolated by adsorption of serum from rabbit E2 to S. sanguis O cells and elution with acetic acid. Arrows indicate the layer of adsorbed S-1 antibody. Marker bar, 0.5 μm.*

outbred animals are repeatedly restricted suggests that the immunogens for these antibodies may in fact possess simple repeating structures. Confirmation of this hypothesis will have to await antigen purification and chemical analysis.

Based on the present data, certain inferences may be drawn regarding the structure of Ag-1. For example, it is possible that Ag-1 from N and O cells both consist of simple repeating units that differ chemically from each other. This

would explain why both antigens elicit a restricted antibody response but would not explain why the antigens react in identity in gel diffusion. It is possible that pleomorphism in *S. sanguis* involves an increase in the number and cross-linking of side chains of Ag-1 that allow a sphere-to-rod transition of the *Arthrobacter* type (11). However, such an altered antigen might be expected to elicit an antibody with increased heterogeneity and might also be different from the antigen of normal cells in gel diffusion. All of these speculations are easily settled by comparing the structure of the purified antigen from N and O cells. Such studies are now underway. On the basis of the data presented we tentatively conclude that there is probably not a great change in the complexity of Ag-1 in O cells.

However, our data do not exclude the possibility that other cell wall components are structurally altered. In fact, the alterations in agglutination and binding of the S-1 antibody argue that there may be significant changes in other cell wall components. The difference in agglutination of N and O cells may be due in part to an alteration in the arrangement of components of the O cell surface. It can be argued that efficient agglutination requires an even distribution of antibody that is lacking in O cells. The changes in the pattern of antibody binding seen by fluorescence and electron microscopy support this hypothesis. The experiments presented in this report offer no clue as to the reason for the altered binding of S-1 antibody. However, there are several possible explanations for this phenomenon which are subject to experimental investigation. (i) It is possible that the antigen is no longer synthesized when cells become pleomorphic. This explanation by itself makes it difficult to explain the gradual appearance of additional bands of fluorescence in grossly pleomorphic cells. (ii) It is possible that the antigen is synthesized and is placed in the wall properly but is masked by other abnormally arranged wall components. Studies of the synthesis and structure of Ag-1 that are now underway may help to decide among these alternatives. It might also prove necessary to study the synthesis of other wall components to answer this question.

Such studies might provide an answer for another puzzle, namely, why is only one antibody eluted from the surface of O cells when that antibody does not completely surround the cells? The most logical explanation at present is that the newly exposed sites on the pleomorphic cell surface are not strongly immunogenic.

Finally, it is noteworthy that the S-1 antibody of *S. sanguis* has several properties in common with an antibody isolated by a similar procedure from *Streptococcus mutans* (8). In both cases, the antibody was isolated by adsorption of crude antisera to whole cells and elution with weak acid. In both cases, the antibody was shown to be a major agglutinating antibody for the cell. Iacono et al. (8) showed that all of the agglutinating activity of crude antisera to *S. mutans* was inhibited by the antigen which reacted with the purified antibody. We found that crude antisera to *S. sanguis* had the same agglutination titer per milligram of IgG as did the S-1 antibody. The antibody isolated by Iacono et al. (8) is group specific for *S. mutans*. Preliminary experiments in this laboratory indicate that the S-1 antibody may be group specific for *S. sanguis* (Eisenberg and Elchisak, unpublished results). Iacono et al. (8) purified the *S. mutans* antigen corresponding to the isolated antibody and showed that it was composed almost entirely of glucose and galactose. Linzer and Slade (13) had previously shown that the group *d* antigen of *S. mutans* consists of glucose and galactose. It will be interesting to compare the chemical structure of purified Ag-1 from *S. sanguis* with its counterpart in *S. mutans*.

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