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Ultrastructural Study of *Chlamydia trachomatis* Surface Antigens by Immunogold Staining with Monoclonal Antibodies

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Surface antigens of *Chlamydia trachomatis* were studied by immunogold staining with monoclonal antibodies and by electron microscopy. The serovar- and subspecies-specific epitopes were the most surface accessible. The species- and genus-specific epitopes were the least surface exposed. Similar serological specificity as that in the microimmunofluorescence test was demonstrated by immunogold staining.

Four groups of *Chlamydia trachomatis* antigens are recognized by immunological analysis of monoclonal antibodies (MAbs) against whole elementary body (EB) antigens by the microimmunofluorescence (micro-IF) test (14, 19), the enzyme-linked immunosorbent assay (ELISA) (1), and the radioimmunoassay (2, 12, 14). The four antibody reaction patterns are serovar-, subspecies-, species-, and genus-specific. The serovar-specific antibodies show the most restrictive activity by reacting with a single serovar antigen. The subspecies-specific antibodies show reactivity against antigens of related groups of serovars. The species-specific antibodies react with all strains of the species *C. trachomatis*. Finally, the genus-specific antibodies show reactivity to both species of the genus *Chlamydia*. To study the surface accessibility of immunological determinants, we used MAbs in the indirect immune electron microscopy assay, using colloidal gold to localize and quantitate epitopes on the EBs of *C. trachomatis*.

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C. trachomatis B/TW-5/OT and H/UW-4/Cx were grown in a HeLa 229 cell culture (9). The organisms were purified on a linear gradient of Renografin (Hypaque-76; Winthrop-Breon, Division of Sterling Drug, Inc., New York, N.Y.) (8). The MAbs used in this study have been described previously (1, 10, 14, 19). The serological specificities and antibody titers of these MAbs are shown in Table 1. All MAbs were immunoglobulin G (IgG). In the immunoblotting test (16) all MAbs with serovar, subspecies, and species specificity reacted with the 40,000-dalton major outer membrane protein (MOMP). The genus-specific MAb CF-2 reacted with the lipopolysaccharide (LPS). The second genus-specific MAb FC-5 did not react with any bands.

TABLE 1. Counts of colloidal gold particles associated with *C. trachomatis* EB in the immunogold stain with MAbs of different serological specificities

MAb			Grain counts for the following Antigen serovar[a]:	
Serological reactivities[b]	Antibody name	Titer[c]	B	H
Serovar				
B	DD-1	800	8.82 ± 4.98 (104)	0.04 ± 0.04 (135)
B	DE-3	400	4.97 ± 2.38 (145)	0.04 ± 0.01 (163)
H	LA-10	1,600	0.06 ± 0.07 (144)	2.52 ± 0.39 (113)
Subspecies				
B, Ba, L2	BB-3	1,600	2.60 ± 1.71 (242)	0.10 ± 0.13 (262)
B, Ba, D, E, G, F, L1, L2	DA-2	3,200	3.39 ± 1.06 (156)	0.01 ± 0.02 (100)
Species	2C-1	1,600	0.67 ± 0.48 (246)	1.81 ± 2.14 (79)
	KG-5	1,600	1.04 ± 0.93 (122)	1.34 ± 0.44 (67)
Genus	CF-2	51,200	0.69 ± 0.64 (100)	1.00 ± 0.80 (121)
	FC-5	6,400	0.44 ± 0.34 (79)	0.26 ± 0.21 (141)
Control	Normal Ascites		0.17 ± 0.24 (202)	0.05 ± 0.07 (98)

[a] Mean number of gold particles/EB ± standard deviation; the number of EBs counted is given in parentheses.
[b] Determined by the micro-IF test.
[c] Determined by the micro-IF test (serovar, subspecies, and species) or ELISA (genus).

The immunogold method for electron microscopy has been described by Faulk and Taylor (6). The procedure used was as follows. A 0.5-ml chlamydia suspension (10^9 particles) was placed in a microfuge tube and pelleted at 12,000 × *g* for 15 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.), washed once with phosphate-buffered saline (PBS), reacted with MAbs (ascites

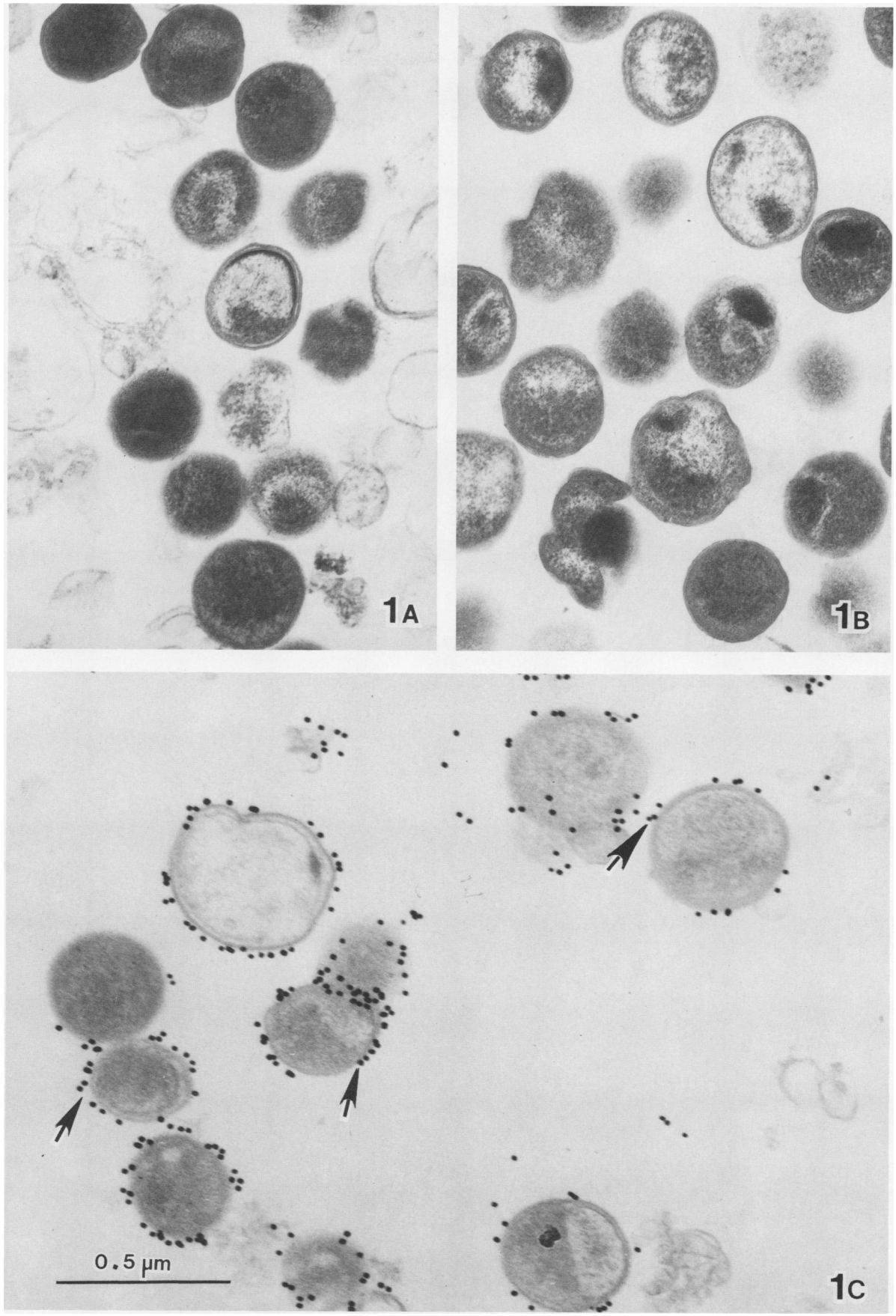

FIG. 1. Electron micrographs showing a serovar-specific epitope detected on the EB surface of *C. trachomatis* by immunogold labeling with MAbs. Purified EBs were reacted with MAbs, followed by incubation with colloidal gold (particle size, 15 nm in diameter)-labeled anti-mouse IgG, fixed with glutaraldehyde, and processed for electron microscopy. (A) Control; reaction of serovar B EBs with normal ascitic fluid. No gold particles can be seen. (B) Heterologous reaction; serovar H EBs reacted with serovar B-specific MAb DD-1. No gold particles can be detected on the EB surface. (C) Homologous reaction; serovar B EBs reacted with serovar B-specific MAb DD-1. Many gold particles are associated with the external surface of EBs (arrows). Lack of contrast was due to omission of lead stain. Magnification, ×60,000.

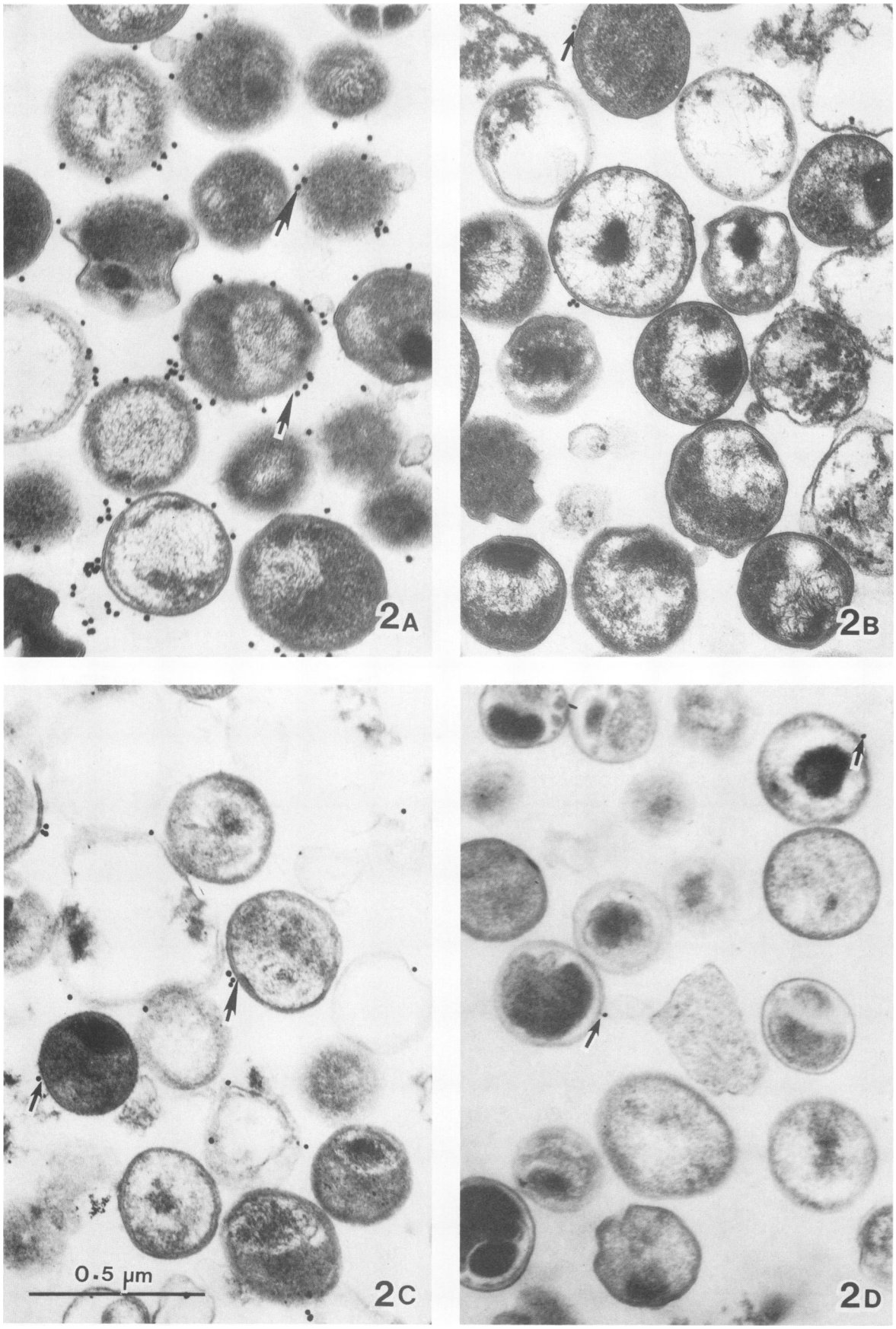

FIG. 2. Electron micrographs illustrating the differences of subspecies-, species-, and genus-specific epitopes detected on the EB surface of *C. trachomatis* by immunogold labeling with MAbs. (A) Reaction of serovar B EBs with subspecies-specific MAb BB-3, which is reactive with B, Ba, and L2 in the micro-IF test. Many gold particles are attached to the EB surface (arrows). (B) Reaction of serovar H EBs with MAb BB-3. Few or no gold particles can be seen (arrow). (C) Reaction of serovar B EBs with species-specific MAb 2C-1, which is reactive with all serovars of *C. trachomatis*. Only a few gold particles are localized (arrows). Gold particles are also seen attached to the cell wall of a cell ghost. (D) Reaction of serovar H EBs with genus-specific MAb CF-2, which is reactive with strains of both species (i.e., *C. trachomatis* and *C. psittaci*). Gold particles are occasionally seen (arrows). Magnification, ×60,000.

fluid) at a 1:100 dilution, incubated at 37°C for 30 min, pelleted, washed twice with PBS, incubated with colloidal gold (particle size, 15 nm in diameter) that was conjugated to anti-mouse IgG (Janssen Pharmaceutica, Piscataway, N.J.) at a 1:15 dilution for 30 min at room temperature, pelleted, washed once with PBS, pelleted, fixed with 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4), and processed for electron microscopic study within 24 h, as described previously (3). Briefly, after at least 2 h of fixation in glutaraldehyde, EBs were washed twice with cacodylate buffer and then fixed in 0.1% OsO_4 in cacodylate buffer for 4 h. EBs were then washed twice with double-distilled water, stained enblock with 0.5% uranyl acetate for 20 min, and embedded in 1% agar. The preparations were dehydrated with a series of graded alcohol and were embedded in Epon 812. Thin sections were cut with a diamond knife and viewed in an electron microscope (Joel 100B) at 60 kV. Randomly selected micrographs were taken at magnifications of ×20,000. At least 10 micrographs were taken from each randomly selected block. Each test was repeated. The colloidal gold particles associated with the external surface of intact EB particles were counted randomly in each electron micrograph. The average number of gold particles per EB was obtained. The counts were analyzed by Student's *t* test.

The electron micrographs of the EB preparations showed that the majority of the chlamydial particles were of the EB form. Small numbers of intermediate and reticulate forms and damaged particles consisting of outer membrane with no cytoplasmic material were also seen. There was little background stain, i.e., gold particles not associated with chlamydiae. Gold particles were seen attached on the outer surface of chlamydiae that had been reacted with homologous antibodies (Fig. 1C). The size of the gold particles was uniform. No gold particles were seen to be associated with chlamydiae that had been reacted with normal ascites (Fig. 1A) or heterologous antibodies (Fig. 1B).

The immunogold staining gave the same reaction patterns as has been observed by the micro-IF (19) and ELISA (1) methods (Table 1). Serovar- and subspecies-specific MAbs reacted with the EBs of their respective serovars B and H, while species- and genus-specific MAbs reacted with both serovars. Grain counts revealed statistically significant ($P < 0.01$) differences in the density among the four serological determinants on the chlamydial EB surface (Table 1 and Fig. 1 and 2). The serovar-specific epitope had the highest density (Fig. 1C). The densities of the other epitopes decreased in order of subspecies-, species, and genus-specific determinants (Fig. 2A, C, and D). The grain counts of species- and genus-specific epitopes were comparable in both serovars B and H.

The immunogold labeling of surface antigens on the native *C. trachomatis* EB revealed differences in the surface accessibility and density among the four serological epitopes. In immunoblots serovar-, subspecies-, and species-specific MAbs recognized the 40,000-dalton MOMP. Because these antibodies reacted with the immunogold stain, it indicates that the MOMP antigens are surface exposed. It further suggests that the serovar and subspecies determinants are in the hydrophilic region (more surface accessible), while the species determinants are in the hydrophobic region (less surface accessible) of the MOMP. Immunogold staining showed that both LPS and non-LPS genus-specific antigens are either nonsurface antigens or are little exposed on the surface of EBs. This finding is consistent with the electron microscopic observation of Dhir and Boatman (5) on the staining of the surface carbohydrate of the chlamydial cell wall with silver methenamine. However, the species- and genus-specific antigens may be rendered more accessible by physical and chemical treatments such as air drying and alcohol or acetone fixation, as in the preparation of EBs on microscope slides for immunofluorescence (15, 17).

The number of grains may also reflect the avidity of the antibody. However, the species- and genus-specific MAbs tested were generally more avid than serovar-specific MAbs, as shown by their higher antibody titers in the micro-IF test and ELISA (Table 1). Therefore, the difference in the grain counts is less, possibly due to the avidity of the antibody.

The immunogold staining results provide some explanations for the phenomenon observed in chlamydial serology. The serology or strain typing by the micro-IF test is based on the endpoint titers and specific cross-reaction patterns (18). Factors that affect the specificity of the micro-IF test are the accessibility and the epitope density of the surface antigens and the antibody titer. For example, genus-specific antigens are as immunogenic as other serological antigens by the fact that an infection with *C. trachomatis* produces a strong genus-reacting antibody that can be detected by the complement fixation test (11) or the indirect immunofluorescence test, with infected cells (inclusion stain) (13) or reticulate bodies (20) used as antigens. However, the genus-reactive antibodies are not a factor in the micro-IF test because the genus-specific antigens are not surface accessible. This is supported by observations that human or mouse antisera do not cross-react with *C. psittaci* EB antigens (7, 17) and that the genus-specific MAbs show weak to no reaction to EBs of both species (14). In the micro-IF test with hyperimmune sera, the highest antibody titers are usually obtained against the serovar-specific antigens (17, 18). The next highest titers are against the subspecies-specific antigens, and the lowest titers are against the species-specific antigens. The differences in epitope densities for these determinants may account for this phenomenon and make the serovar determination possible. This factor may be more critical for differentiating broadly cross-reacting serovars like B, Ba, D, and E.

Clark et al. (4) have shown two binding patterns of MAbs to chlamydial surface antigens by immunoferritin labeling. The neutralizing antibodies bind to the outer membrane surface in a homogenous pattern, while the non-neutralizing antibodies showed an irregular binding pattern. However, in our study no distinct binding patterns were shown between neutralizing (DD-1, LA-10, DA-2) and non-neutralizing (DE-3, BB-3, 2C-1, KG-5, and FC-5) antibodies (10). The distribution of colloidal gold particles on the EB surface was random with both neutralizing and non-neutralizing antibodies.

We have shown that immunogold staining with MAbs is a useful tool for studying chlamydial surface antigens. It provides a means for studying the location and epitope density of specific surface antigens and the immunochemical nature, serological specificity, and biological function of the antigens.

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