Ultrastructural Study of the Interaction between *Acholeplasma laidlawii* and Antibody

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The ultrastructure of agar-grown *Acholeplasma laidlawii* incubated with specific antiserum or IgM fractions of this antiserum has been investigated by the thin-sectioning technique. Antiserum treatment resulted in the development of giant cells along the colony circumferences and in the coating of normal-size mycoplasmas with a periodically arranged extramembranous layer, consisting of attached immunoglobulins as shown by indirect immunoferritin labelling. The regular structure of the coat was not influenced by changes in temperature or by fixation of the membrane antigens prior to reaction with antibody. Extracellular enveloped viruses were uniformly covered with antibody in these experiments. IgM fractions of antiserum in high concentrations produced a similarly uniform extramembranous layer both on mycoplasmas and viruses. Possible explanations of the difference demonstrated between the regular arrangement of antigenic determinants on *A. laidlawii* membranes and the previously observed uniform binding of immunoglobulins to *Mycoplasma gallisepticum* are discussed.

INTRODUCTION

Micro-organisms in the class *Mollicutes* (Edward & Freundt, 1967) are distinguished from other prokaryotes by, among other things, their lack of a true cell wall, a property which makes these organisms well suited for studying the prokaryote cytoplasmic membrane. In a recent report (Vinther & Freundt, 1979), some electron microscopical observations of the reactions of specific antibody with the cytoplasmic membrane of *Mycoplasma gallisepticum* were described. The present investigation constitutes a more extensive study of the ultrastructural aspect of the interaction between another membrane-delimited prokaryote, *Acholeplasma laidlawii*, and antibody, in which the effects of temperature, fixation, immunoglobulin class and other factors are assessed. *Acholeplasma* species differ from *Mycoplasma* species in not requiring cholesterol for growth and in containing much less cholesterol in their membrane (Razin, 1978). In this respect *Acholeplasma* membranes show a closer relationship to bacterial cell membranes than do *Mycoplasma* membranes.

METHODS

Organisms and growth conditions. The strains used were the type strain of *Acholeplasma laidlawii*, PG8, and three wild strains C1121, C1173 and C1201, isolated from a cow, a buffalo and a goat, respectively, and identified as *A. laidlawii* by the growth inhibition test and immunofluorescence at the FAO/WHO Collaborating Centre for Animal Mycoplasmas, Aarhus, Denmark. The organisms were grown in liquid or on solid B medium (Ernø & Stipkovits, 1973) at 37 °C. In some experiments, antiserum was incorporated in the solid growth medium.

Antisera. Rabbit hyperimmune serum against *A. laidlawii* PG8 was produced essentially as previously described (Black, 1973). Serum derived from samples taken before immunization was used as a control. Two pools of hyperimmune serum from two different rabbits, showing high titres when tested against PG8 antigen by the growth inhibition (Black, 1973) and metabolism inhibition (Purcell *et al.*, 1966) methods, were used

in the study. The corresponding pre-immune sera showed no detectable reactions in the two tests. The sera raised against PG8 were as potent against the three wild strains of *A. laidlawii* in the growth inhibition test as they were against the homologous antigen. All sera were heat-inactivated (56 °C, 45 min) before use in the experiments.

Serum fractionation. One of the two antisera and the corresponding pre-immune serum were fractionated on a column of Sephadex G-200 using 0.1 M-Tris/0.2 M-NaCl, pH 8.0, as elution buffer. The peak fraction and higher molecular weight fractions of the first peak that emerged, which contained the IgM immunoglobulins, were pooled, reduced to the original volume by negative pressure dialysis, and dialysed against 0.1 M-Tris buffer, pH 7.4. The resulting IgM fractions were compared with whole serum by the growth inhibition method.

Antibody treatment. *Acholeplasma laidlawii* cells were incubated with specific antibody either during growth of the organisms on solid medium or after removal of colony-containing cylinders of agar from the solid medium and transfer to test tubes holding the appropriate antibody dilutions. In the former experiments, antiserum was added to the agar plates by filling a central hole, punched out with a Pasteur pipette, with 15 µl undiluted serum. Serum was allowed to diffuse radially for 3 d at 4 °C, and then the plates were inoculated with a dilute liquid culture of the organisms. Subsequent incubation at 37 °C was for 4 to 5 d, until colonies near the central hole had reached a diameter of at least 100 µm.

Most antibody reactions, including those with IgM antibody, were carried out on small agar cylinders (1 mm diam. × 4 mm height) removed from the solid medium with a Pasteur pipette and containing on their surface one or two colonies. Whole antiserum was used in the dilution 1:40 in 0.1 M-Tris buffer, pH 7.4, and the incubation time was 1.5 h, whereas incubation with IgM antibody, either undiluted or diluted 1:10 in Tris buffer, was for 3 h. In some experiments, antibody treatment was preceded by fixation of the colonies *in situ* with 0.3 % (v/v) glutaraldehyde in veronal-acetate buffer, pH 6.1 (Ryter & Kellenberger, 1958) as previously described (Vinther, 1976). The fixation was stopped by gently washing the agar plates with Tris buffer. To study the effect of temperature on antibody binding, incubations were carried out both at 4 °C and 37 °C; otherwise they were done at room temperature. Excess antibody was removed by washing the agar cylinders three times with Tris buffer (at 4 °C, 37 °C or room temperature) with gentle agitation.

Control experiments, in which pre-immune serum was substituted for rabbit immune serum in the same dilution, were always carried out in parallel.

Indirect immunoferritin labelling. Indirect immunoferritin labelling of organisms of strain PG8 was performed as described by Vinther & Freundt (1979). The cells were prefixed with 0.3 % (v/v) glutaraldehyde before incubation with rabbit immune serum diluted 1:40 in Tris buffer. The label used was a ferritin-conjugated gamma globulin fraction of goat anti-rabbit IgG (Miles-Yeda, Israel) diluted 1:40 in Tris buffer.

Tellurite reduction. To investigate the effect of antibody treatment on tellurite reduction, unfixed colonies of *A. laidlawii* PG8 grown on solid medium containing antiserum were incubated with 0.1 % (w/v) K₂TeO₃ in distilled water as described by Vinther & Freundt (1977). Incubation with the reagent was continued until a dark coloration of colonies near the antibody reservoir could be detected in the light microscope (about 3 h) and colonies were subsequently processed for electron microscopy as described below.

Electron microscopy. Details of the procedure used in the preparation of cells for electron microscopy have been described previously (Vinther, 1976). Fixation with 0.3 % glutaraldehyde was omitted in experiments with IgM antibody and in part of the investigation of the effects of temperature and fixation on antibody binding, apparently without appreciable ultrastructural effects. Final fixation with 3 % glutaraldehyde was generally carried out at room temperature, but in experiments on the effect of temperature this fixative was used at 4 and 37 °C. In the preparative procedure used for tellurite-treated cells, heavy metal fixatives and stains were omitted (Vinther & Freundt, 1977). Thin sections were examined in a Jeol JEM 100B electron microscope either unstained or after staining with lead citrate or magnesium uranyl acetate or both.

RESULTS

Colony and cell morphology after antibody treatment

Growth of all four strains of *A. laidlawii* on solid medium containing antiserum against strain PG8 was partially inhibited in a distinct zone around the antibody reservoir. The colonies within such zones were smaller and less crowded than those outside. Also, in the light microscope, these colonies showed an irregular undulating periphery.

The most conspicuous effect of antibody incubation seen in the electron microscope was the development, mostly along the periphery of colonies, of cells with diameters as large as 3 to 4 µm (Fig. 1) and containing rather loose cytoplasm. Their limiting membrane appeared intact. The intercellular space in this region of the colonies was filled with amorphous

Fig. 1. Thin section showing the peripheral part of an *A. laidlawii* colony (strain PG8) grown on solid medium containing antiserum. Huge cells with loose or condensed cytoplasm are evident. The intercellular space is filled with amorphous precipitate. Bar marker represents 1 μ m.

Fig. 2. Section from a colony (strain PG8) grown on unsupplemented medium. One mycoplasma cell and several spherical virus particles are seen. Both types of organisms appear to be limited by a triple-layered membrane. A thin coat of extramembranous material lines the mycoplasma cell membrane. Bar marker represents 200 nm.

Fig. 3. Cells of strain C1201 grown on medium containing antiserum. Cells are covered with a layer of antibody which varies periodically in density (arrows). Bar marker represents 200 nm.

Fig. 4. Section of the membrane of a cell (strain PG8) grown on medium containing antiserum, showing a regular spacing between antibody attachment sites. Bar marker represents 100 nm.

Fig. 5. Cells of strain PG8 fixed with 0.3% glutaraldehyde prior to incubation with specific antiserum. A periodically arranged (arrows) extramembranous coat is observed. Bar marker represents 200 nm.

material, presumably precipitated antibody. Giant cells were not observed in colonies grown on medium supplemented with pre-immune serum as a control, nor were they seen after incubation of colonies with antiserum following growth on unsupplemented medium. However, the gross morphology of the majority of cells grown in the presence of antiserum did not appear to differ from that of cells grown on medium supplemented with pre-immune serum or on unsupplemented medium. Cells grown under the latter conditions displayed the characteristic triple-layered outer boundary with only a very narrow extracellular coat, if any (Fig. 2). In addition, thin sections of all four strains examined showed the existence of varying numbers of small spherical particles, with diameters ranging between 40 and 100 nm, in the space between the mycoplasma cells (Fig. 2). The interior of these cells either showed relatively high contrast or looked nearly empty. The particles were clearly delimited by a

triple-layered membrane resembling that of the mycoplasmas. Cells grown on medium containing pre-immune serum did not visibly differ from cells grown on unsupplemented medium.

The only ultrastructurally detectable effect of antibody treatment, besides the formation of giant cells, was the development of a thick external coat both on mycoplasmas grown in the presence of antiserum and on cells incubated with the serum for shorter periods after growth had been stopped. The average thickness of the extracellular layer varied, but was generally in the range of 10 to 40 nm. On the majority of cells seen in thin sections the layer was arranged in a periodic way with regular changes in the thickness of the coat emerging from the outer profile of the triple-layered membrane (Fig. 3). The period length measured was 15 to 40 nm, variation occurring from cell to cell and also on different membrane areas of the same cell. A similar periodicity in the arrangement of the external coat observed on the small intercellular particles could not be ascertained and was not present in the loosely arranged material surrounding the giant cells. On some of the mycoplasma cells additional ultrastructural features of the antibody coat could be discerned on membrane areas with a high degree of curvature. As shown on Fig. 4, the points of attachment of the extracellular antibody layer to the outer membrane track were separated by a regular spacing, leaving pockets without adhering material close to the membrane. The superficial part of the coat appeared nearly confluent. The characteristics described for the binding of antibody to *A. laidlawii* were observed on all four strains examined, using either of the two antisera raised against strain PG8, but were not seen on cells incubated with pre-immune serum.

Influence of fixation and of incubation temperature

To study the possible effects of prefixation with glutaraldehyde on the antibody binding pattern, colonies fixed *in situ* with 0.3% glutaraldehyde were incubated with specific antiserum. The experiments did not show any changes in the arrangement of the extracellular coat as a consequence of the attempted immobilization of the membrane antigens. A definite periodicity of the antibody layer, with a period length of the same magnitude as that seen on unfixed cells, was also observed on prefixed cells (Fig. 5). Incubation with antibody of unfixed cells and subsequent fixation with 3% glutaraldehyde at 4 °C also failed to reveal any effect on the organization of the antibody coat when compared with incubation of previously unfixed cells at 37 °C.

Reaction with IgM antibody

Incubation of unfixed colonies with the undiluted IgM fraction of serum antibodies resulted in coating of the mycoplasma cells with a relatively uniform layer of attached immunoglobulins, 20 to 30 nm thick (Fig. 6). A similar coat was also observed on the small extracellular particles. A periodic arrangement, like that described on the mycoplasmas incubated with diluted whole serum, could not be detected on either the extracellular particles or the mycoplasma cells, although occasionally a small number of superficial, apparently equally spaced, spikes were observed on the IgM-treated mycoplasmas. Treatment with IgM fractions diluted 1:10 gave rise to a considerably thinner layer of extra-membranous material, and in control tests with IgM from pre-immune serum only insignificant amounts of adhering precipitate were observed.

Indirect immunoferritin labelling

Indirect immunoferritin labelling of strain PG8 confirmed that the material seen attached to the membrane of *A. laidlawii* following incubation with specific antiserum consisted of immunoglobulin molecules. However, the arrangement of the electron-dense ferritin particles around labelled cells (Fig. 7) did not reflect the periodic binding of antibody to the cell membrane described above, and the amorphous coat visible on the cells appeared rather

Fig. 6. A cell of strain PG8 incubated with IgM antibody, showing a thick and relatively uniform extracellular layer. Bar marker represents 200 nm.

Fig. 7. Indirect immunoferritin labelling of a cell of strain PG8. A uniform extramembranous layer is decorated with apparently randomly distributed ferritin particles. The attached virus particle (arrow) is also labelled with ferritin. Section-staining omitted. Bar marker represents 200 nm.

Fig. 8. Control cell (strain PG8) incubated with pre-immune serum and ferritin label. No extramembranous immunoglobulin layer is present, and there is only sporadic attachment of ferritin particles (arrow). Section-staining omitted. Bar marker represents 200 nm.

homogeneous. Ferritin labelling of the small extracellular particles was also present (Fig. 7), whereas intracellular ferritin was not observed, except occasionally in damaged mycoplasma cells. Cells incubated with pre-immune serum as the first reagent displayed essentially no membrane coat and only sporadic attachment of ferritin particles (Fig. 8).

Tellurite reaction

The result of histochemical treatment to reveal membrane-located tellurite-reducing activity of organisms incubated with antibody showed that membranes surrounded by an amorphous antibody coat were still capable of reducing the substrate.

DISCUSSION

Occasional findings of huge mycoplasma cells in cultures grown under ordinary conditions have been reported (Anderson & Barile, 1965; Allen *et al.*, 1970). In the present investigation, however, the formation of such cells was an invariable consequence of growth on solid medium containing antiserum but not on medium supplemented with pre-immune serum, thus ruling out the possibility of a purely osmotic effect. Giant cells were not observed in *M. gallisepticum* 'break-through' colonies in immune growth inhibition zones (Vinther & Freundt, 1979).

The small, apparently membrane-bounded, extracellular particles detected in thin sections of all four strains of *A. laidlawii* closely resembled the previously described mycoplasma viruses MV-L2 (Gourlay *et al.*, 1973; Liška & Tkadleček, 1975). Rod-like MV-L1 viruses, as depicted by Milne *et al.* (1972), were not seen in any of the preparations.

The development of a periodically arranged extramembranous antibody layer on treatment of *A. laidlawii* organisms with specific antiserum is in contrast to the results obtained after corresponding incubations of *M. gallisepticum* cells with antibody (Vinther & Freundt, 1979), where the antibody coat appeared almost uniform in thickness. The apparent variations in period length shown in thin sections of *A. laidlawii* cells may be reflections of a purely geometrical effect caused by variations in the sectioning angle relative to the lattice axes of the supposedly regular pattern of antigenic sites on the membranes. The better resolution of the antibody coat on the more curved membrane areas may be a consequence of this curvature, reducing overlap in thin sections. From examination of such membrane areas it is deduced that there is a definite spacing of the antigenic sites on *A. laidlawii* membranes of about 25 nm.

To investigate the lateral mobility of the mycoplasma membrane antigens, antibody binding at 4 and 37 °C was compared directly: no alteration in the regular binding pattern was observed. Biotin-avidin-ferritin labelling of outer membrane proteins of *A. laidlawii* has demonstrated that reversible clustering of the proteins occurs in a temperature range corresponding to the lipid phase transition between the smectic and paracrystalline states, whereas the proteins are dispersed outside this range (Wallace *et al.*, 1976). Freeze-fracture studies of inner membrane particles (James & Branton, 1973), on the other hand, showed aggregation of membrane proteins at temperatures near and below the transition point. Clustering of the antibody label was not detected in the present study, but since the transition temperature of the membranes used was not known, it cannot be decided whether the regular arrangement of membrane antigens at both 4 and 37 °C was a property of the smectic or the paracrystalline states or both.

Electron microscopic examination of antibody labelling of membranes on cells previously fixed with glutaraldehyde demonstrated that the observed periodic distribution of membrane determinants was not produced by the binding of immunoglobulin molecules to these determinants thereby causing a redistribution of the membrane antigens. It should also be noted that the observed binding pattern was independent of the state of metabolism of the organisms during antibody treatment, since the membrane coat had the same appearance, whether formed during growth of the organisms in medium containing antiserum or produced after growth had been stopped by glutaraldehyde fixation or cooling to 4 °C. Since only a relatively thin extramembranous coat was detected when the cells were incubated with IgM fractions diluted 1:10, the periodically arranged coat on cells incubated with whole serum (1:40) was probably made up of the smaller immunoglobulins, in particular IgG. The efficient labelling of mycoplasma cells and viruses incubated with antiserum with ferritin-conjugated anti-rabbit IgG also suggested that the main component of the attached antibody layers in such experiments was specific IgG.

Several explanations for the apparently higher degree of organization of the antibody coat on *A. laidlawii* membranes as compared to *M. gallisepticum* membranes (Vinther & Freundt, 1979) may be given. One major difference observed between the two species in the electron microscope was the heavy infection of all strains of *A. laidlawii* with spherical viruses, which may perhaps have modified the membrane of their host in such a way as to produce the regularity in surface determinant structure. Whether or not these determinants on the cell membranes were directly virus-specified could not be decided, since all four mycoplasma strains examined were infected with (the same) virus and since the antisera used were raised by an antigen containing both mycoplasmas and virus particles. The potential effect of mycoplasma virus interaction should be taken into consideration in the interpretation of membrane experiments, especially with *A. laidlawii*. Another factor which may influence the architecture of mycoplasma membranes relates to the different ways in which membrane fluidity is regulated by changes in cholesterol incorporation in *Mycoplasma* species or by variation of the fatty acid content in the *A. laidlawii* membrane (Razin, 1978). It is possible that the observed uniformity of the membrane coat on *M. gallisepticum* was a consequence

of this membrane being in a less viscous state under the experimental conditions. Finally, the chemical nature of the antigens detected in the two species may have differed, although it is conceivable that the antigenic determinants on membranes of both species were mainly of protein nature (Kahane & Razin, 1969).

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