Morphology and Viability of Large Bodies of Streptococcal L-Forms

DAVID J. BIBEL* AND JOHN W. LAWSON

Department of Dermatology Research, Letterman Army Institute of Research, San Francisco, California 94129,* and Department of Biology, University of Missouri, Kansas City, Missouri 64110

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A procedure is described for the massive formation and isolation of large bodies of group A and D streptococcal L-forms. Up to 90% individual bodies of 20 to 100 μm in diameter can be produced in pour plates of nutrient gelatin and subsequently harvested by simple micromanipulation. The growth of these giant bodies was followed by light microscopy and their ultrastructure and internal architecture was examined by transmission and scanning electron microscopy. Large bodies had a honeycomb structure of vacuoles. Numerous small units could be observed inside, protruding into, and between vacuoles. Intact large bodies (30 to 60 μm), when placed in small amounts of broth and incubated at 35 C, initiated turbid cultures, but when set on agar, they ruptured, releasing internal granules and producing typical L-form colonies. The number of internal colony-forming units (CFU) was correlated with the size of large bodies. Up to 200 CFU were detected in bodies of 40 to 60 μm in diameter, whereas corpuscles of 20 to 30 μm averaged only three CFU. The inefficiency of replication was apparent with the determination by light and electron microscopy that at least 100 times as many granules and elementary corpuscles as CFU were produced inside large bodies.

The mode of reproduction of L-forms of bacteria is still a subject of debate. Many mechanisms have been described, such as budding (1, 4, 6, 10), fragmentation (4, 7), and fission (1, 3, 4), and it is quite possible that, depending on environment, L-form variants may undergo some or even all proposed processes during growth. The role of large bodies, granules, and elementary corpuscles in the reproductive cycle is just as unsettled today as it was 20 years ago. The development of large bodies from granules has been observed (3, 7, 11, 13), and some investigators (3, 11, 13) have regarded the form to be an end cell, at least in broth. Other workers (6, 7, 8, 10) suggest that large bodies, as part of an alternate cycle of reproduction, release numerous elementary bodies and granules which can follow the usual process of fission or budding.

Whether the intact large body itself or its internal subunits are viable has not been satisfactorily demonstrated. Roux (14), by testing isolated agar-grown large bodies, concluded that the forms were not capable of reproduction. Dienes (7) and Green et al. (10), among others, have observed by light microscopy the release of smaller units from vacuoles but have not substantiated their viability.

To ascertain the reproductive capacity of the various L-form corpuscles, physical methods of separation, such as filtration (5, 17), have been attempted with limited success. This report presents a unique system for the formation, recovery, and isolation of large bodies of great size. In addition, the interior of large bodies is explored by light, scanning electron, and transmission electron microscopy. By separating individual large bodies from any surrounding granules and elementary corpuscles, we have been able to demonstrate the viability of internal bodies.

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MATERIALS AND METHODS

Microorganisms. L-form cultures of strains ADA and GL8, group A streptococci, and of strain F24, a group D streptococcus, were kindly supplied by R. M. Cole and H. Gooder, respectively. We also used an L-form which we had derived from lysozyme-induced protoplasts of Streptococcus faecium strain F24 (4). All L-form variants are stable and have been subcultured in broth for several years. Stock cultures were maintained at -70 C and transferred every 6 to 8 months. Before the current investigation we had conducted some serological and biochemical studies which, as a result, verified the common origin of our strain and that supplied by H. Gooder, but did show certain subtle differences here and in

Media. Media for the routine maintenance and growth of the above bacteria have been described in earlier reports (3, 4). The test medium used for the production of large bodies was composed of 25% (wt/vol) gelatin (Difco) and Trypticase soy broth (BBL) with either 0.6 M sucrose, 0.5 M NaCl, 0.43 M NH₄Cl, or 7% (wt/vol) polyvinylpyrolidone 4000 (Sigma) as the osmotic stabilizer. At times 10% heat-inactivated horse serum was added to each medium to enhance growth. Viability of large bodies was tested in broth consisting of Trypticase soy broth and 0.43 M NH₄Cl and on similar agar medium containing 1.2% agar, reagent (Oxoid).

Formation of large bodies. Aliquots of 0.1 ml, taken from 10-fold dilutions of a 1- to 2-day broth culture, were added to gelatin plates which were previously liquefied by heating at 37 C for 30 min. After gentle mixing with a pipette or rod to avoid bubbles, the medium was allowed to solidify at refrigeration temperature with care to ensure relatively uniform thickness. Plates were incubated in sealed containers (anaerobe jars worked well) at room temperature (below 25 C).

Recovery and isolation. Usually after 3 to 5 days, blocks (10 by 5 cm) of gelatin were cut and removed with a sterile Bard-Parker blade and placed in a sterile plastic Rodac plate. These blocks, which contain from one to several hundred large bodies, were flooded with a sterile solution consisting of 0.5 M NaCl or 0.43 M NH₄Cl, 0.05 M phosphate buffer, pH 7.2, and 100 μg of gelatinase per ml (Nutritional Biochemistry Co.). After heating at 37 C for 30 to 45 min, the plate was swirled gently to dissolve any residual gelatinous clumps. Intact large bodies (observed at ×100) were recovered from the resulting suspension by use of sterile, hand-held micropipettes. The L-form bodies were washed, individually or in groups, at least six times by serial transfer into 0.5-ml drops of buffer located in a second Rodac or petri dish.

Microscopy. Photographs from light microscopy were obtained initially with a Polaroid ED-10 camera and type 107 pack film. Kodak high-contrast copy film was used at times to accentuate granules and membranes. General preparative techniques for scanning electron microscopy (SEM) have been described in a previous report (2). Gelatin blocks were hardened by fixation and critical-point drying. By fracturing the block with a razor blade, we were able to observe split large bodies on the exposed edges. In one experiment, glutaraldehyde-fixed large bodies, instead of being dried by the usual critical-point method, were frozen in liquid nitrogen and freeze-dried with a Pearse tissue dryer for 12 h at −60 C. Photomicrographs were taken with Polaroid film type 55 P/N with a Cambridge Stereoscan electron microscope operated at 20 kV.

Specimens for transmission electron microscopy were prepared as follows. One-millimeter blocks of solidified gelatin containing L-form growth were cut and prefixed for 1 h in a mixture of 5% glutaraldehyde, 0.1% ruthenium red, and 2% NH₄Cl in 0.1 M cacodylate buffer (pH 7.3). The blocks were then washed repeatedly with a 0.1% solution of ruthenium red in cacodylate buffer. Specimens were then postfixed with 1% OsO₄ in buffer with 1% ruthenium red for 1 h, washed four times in distilled water, and stained (en bloc) with saturated (aqueous) uranyl acetate for 1.5 to 2 h. The blocks were dehydrated with ethanol by the usual procedure and then embedded in Spurr low viscosity embedding medium using the hard formula. Ultrathin sections were stained with uranyl acetate and lead citrate. Photomicrographs were obtained using a Seimens 1A electron microscope.

A second method was used for isolated bodies. After harvesting large bodies from gelatin and washing them three times in 0.05 M phosphate buffer, pH 7.2, containing 3% (wt/vol) NaCl, they were fixed with 2.5% (vol/vol) glutaraldehyde in buffer for 1 h. This and all subsequent operations involved micro-manipulation with specimens viewed at ×40 or ×100. Next, bodies were washed in the osmotically stabilized phosphate buffer and then placed in buffered 1% (wt/vol) OsO₄ for 1 h after a rinse in 0.1 M sodium acetate buffer, pH 3.9. Then the bodies were washed again in the sodium acetate solution, dehydrated in graded ethanol solutions, and embedded in Spurr low viscosity resin mixture. The uranyl acetate and lead citrate stained sections were viewed in a Hitachi electron microscope.

RESULTS

Formation and growth of large bodies. Of the various stabilizers examined, NH₄Cl by far gave the best yield of large bodies for all strains tested. Less effective was NaCl, with sucrose and polyvinylpyrolidone being completely unsatisfactory, allowing no induction of large bodies. Horse serum enhanced early growth but somewhat clouded the media, interfering with the clear observation of smaller L-form bodies. Its use was discontinued. No appreciable differences in yields were found among test strains, but group D L-forms were generally more consistent in large-body formation.

That large bodies were not merely selected from the broth for continued growth is suggested by the following. When observed at ×100, three times the number of large bodies was seen on day 2 than 24 h earlier, and by day 5, we found 10 times the original number. Furthermore, when both gelatin and agar media were inoculated with dilutions of a broth culture, virtually 100% agar colony-forming units (CFU) developed into foci of large bodies inside gelatin.

In the initial studies horse serum was added to broth cultures. Approximately 5% of these cells, after subculture in gelatin, were found to be individual large bodies of about 10 μm in diameter. The remainder were clusters of two to ten vacuolated large bodies; many appeared
to be budding. Clustering in broth was avoided by the removal of serum from cultures. Separation into individual L-form units was verified by microscopy before inoculating gelatin plates. This greatly altered the results, for at 2 days about 90% foci were individual large bodies of 3 to 5 μm in diameter. By 4 days their size ranged from 15 to 25 μm with 10% apparently budding. Clusters of two to three units remained at 10 to 15% at day 5, with large bodies now of 15 to 30 μm in diameter. (Ultimately the average intact large body reached 50 μm. Giant forms of more than 100 μm were often isolated.)

No differences in yield or growth of large bodies were found using inocula in lag, exponential, or stationary phase. Growth was tested in gelatin medium incubated at 35 C instead of the usual room temperature. At first colonies of small granules appeared in the viscous solution; by 48 h only colonies of large bodies were detected. Thus, the gelatin medium itself seems to be the responsible factor with its physical state playing, at most, a secondary role.

When introduced to shearing forces, membranes were easily broken. Tearing occurred even when gelatin blocks were melted in the absence of the surrounding buffer solution. Upon rupture of the external membrane, large bodies in buffer expanded in diameter and lost both their even outline and their optical density. Subunits, separated by this manner, were loosely held together in a spherical array.

Large bodies with few vacuoles were somewhat more resistant to rupture and could be subjected to repeated poking and compression by microprobes. Age was an important factor as 1-week-old large bodies were more fragile than those of 3 to 4 days of incubation.

**Morphological studies.** The growth in gelatin medium of L-form variants to vacuolated large bodies of great size was followed by light microscopy for 2 weeks. Figure 1 shows selected forms which represent the apparent sequential development of giant bodies. Large bodies were first detected after 24 h and almost always contained three vacuoles. By day 3 the number of vacuoles increased to five or more. As the body increased in diameter, the creation of vacuoles accelerated and could no longer be tallied. With apparent continuing synthesis of internal membranes, the large bodies became optically dense. However, large bodies situated at the surface of the gelatin developed into foamy colonies of large bodies (Fig. 2). Among the test strains the only discernible variation was the slightly increased optical density of the group D L-forms.

SEM showed a large body of about 30 μm in diameter to possess a smooth exterior membrane (Fig. 3) in contrast to the rough surface of smaller sized bodies in broth (3). The internal architecture as seen within the split bodies embedded in hardened gelatin was of particular interest. Figure 4 illustrates the honeycomb

![Fig. 1. Formation of giant bodies of groups A and D L-form streptococci within gelatin medium. Representative forms show sequential development. ×550. (a) 1 day, (b) 2 days, (c) 3 days, (d) 4 days, (e) 5 days, (f–j) 6 days, (k) 7 days.](http://iai.asm.org/)

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structure of vesicles. Numerous convex units as small as 0.05 μm were seen protruding into the vesicles, and separate, spherical granules of 0.2 to 0.6 μm were also clearly noted (Fig. 5, 6). Other photomicrographs showed granules of 0.2 μm between membranes of adjacent vesicles (Fig. 7).

Since neither light nor SEM could prove that the numerous vacuoles were contained within a single membrane and that the gelatin pour plate did allow formation of large bodies rather than compressed colonies of small corpuscles, transmission electron photomicrographs were required. Figure 8 presents morphological evidence of a uniting membrane. Figure 9 displays the outer membrane of the same cell at higher magnification. A typical membrane can be seen.

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**Fig. 2.** Foamy L-form colony of strain GL8 at surface of gelatin. ×550.

**Fig. 3.** Surface of a strain ADA-L large body of 35 μm in diameter viewed by SEM. Body rests on Nucle-pore membrane.

**Fig. 4.** Split large body of group A streptococcal L-form strain GL8-L. Note honeycomb arrangement of vesicles. ×2,000.
surrounding the body and the internal vacuoles. However, the possibility still existed that the harvesting procedures selected colonial or cluster forms. Electron microscopy confirmed that isolated elements were individual large bodies (Fig. 10). Some vacuoles at the periphery were somewhat collapsed.

Photographs were interesting in that they displayed in one L-form unit features which have been observed at times in many different bodies and preparations by other investigators: formation of internal optically dense bodies, budding into vesicles, and triple membranes. The incorporation and location of chromosomal material remained obscure.

Viability studies. To determine whether intact large bodies were viable, we selected five bodies of about 30 to 40 μm in diameter, washed each 10 times, and placed each in a microtiter well containing 0.2 ml of broth. Two samples of buffer surrounding each large body served as controls, since undetected, viable granules might be carried along during the washing process. All control broths remained sterile, but four of the five large bodies produced turbid cultures when incubated at 35 C for 24 h. The fifth large body was found intact. With vigorous agitation the cell was ruptured and by the next day the broth was turbid with growth. The
mode of replication remained obscure. On three occasions when an isolated large body of 30 to 40 \( \mu m \) in diameter was maintained at room temperature for 18 h in a sealed, hanging drop of broth, the body remained intact with no obvious growth or replication.

In a second approach 10 isolated bodies were placed on the surface of agar media. Again control supernatants of buffer were used. All large bodies ruptured when the supporting fluid was absorbed by the agar, and typical L-form colonies were present after incubation at 35 C. At times the internal granules would spread upon release and produce several satellite colonies.

The last test of this series was to reintroduce large bodies into gelatin medium. Two bodies of 20-\( \mu m \) diameter were chosen to permit continued expansion. Indeed, one body grew larger, to 50 \( \mu m \) for 4 days. The second probably tore open, for 11 foci were found with one occurring as a cluster colony.

A 30-\( \mu m \) diameter body was placed between a sterile slide and cover slip and crushed. We noted a release of internal units from one area, perhaps the point of membrane tearing (Fig. 11). The smooth, continuous outline was lost, and within 1 min the body had lysed leaving debris and more internal granules and vacuoles. The material was carefully harvested and inoculated upon agar; L-form colonies were found after incubation.

We attempted to correlate size of large bodies and number of internal granules with CFU. Viable units should increase with larger populations of released granules. The results from 21 isolated large bodies are listed in Table 1. As breakage of the membrane was achieved by crushing or cutting of the body followed by tearing of the debris, multiple membrane bound granules may have remained united and produce only one colony. Although ruptured membranes appeared to disintegrate rapidly, these values are probably minimal.

The number of released CFU seemed far less than the total number of internal granules. To attain an estimate of the total production of granules, photographic analysis by light microscopy and SEM was utilized. A large body measuring 30 \( \mu m \) was crushed between a slide and cover slip and viewed at \( \times 1,000 \). The vast majority of the approximately 200 liberated bodies were around 0.5 \( \mu m \) in diameter. Only six were above 1 to 2 \( \mu m \). A second large body of 45 \( \mu m \) was placed on agar and photographed before complete disintegration could occur (Fig. 12).
The unit consisted of about 500 vesicles of 2 to 20 \(\mu m\) and at least 300 optically dense granules. Almost all granules were located in areas between and along the inner edge of vacuoles. Eighty-five units measuring 1 to 2 \(\mu m\) were counted.

Of interest was an internal body of 10 \(\mu m\) which in turn contained one body of 4 \(\mu m\), one of 3 \(\mu m\), and at least five below 1 \(\mu m\) diameter. One basic problem in estimating total numbers of granules was the limitation in resolution of light microscopy, resulting in the absence from tallies of units below 0.3-\(\mu g\) diameter. When glutaraldehyde-fixed large bodies were freeze-dried, they fragmented but remained in somewhat confined areas. Photomicrographs of these specimens by SEM showed that many granules were membrane bound, making enumeration difficult. It was estimated that a 30-\(\mu m\) large body contained at least 500 granules.

The smallest separate spheres detected were 0.2 \(\mu m\) in diameter. From these studies the inefficiency of replication became increasingly apparent. It seems that less than 1/100 of granules are capable of developing into a colony.

**DISCUSSION**

Scanning electron micrographs showing the arrangement of interior membranes of large bodies offer a new perspective in studying the ultrastructure of the L-form. Triangular junctions (Fig. 5) have been also observed by Cole (6). Holes seen on membranes are probably artifacts of splitting open the body, but may possibly be of structural or physiological significance.

The variation in size and convexity of the small units protruding into the vesicles of giant bodies as seen by SEM is suggestive of a progression of evagination of granules. Such a con-

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**FIG. 8.** Electron photomicrograph of an L-form large body of *S. faecium* strain F24 grown in gelatin medium. The section measures approximately 30 \(\mu m\) in diameter. \(\times4,200\).
cept has been described by Cole (6) and van den Hoof and Hijmans (12). The mechanism for the formation of vacuoles, of granules inside vacuoles, and of granules entirely surrounded by the cytoplasm of the large body has not been adequately explained. It is reasonable that the process of membrane invagination or evagination may be responsible and may also account for budding, fission, and fragmentation. Whether one process or another occurs is probably dependent on a wide variety of factors including flexibility of membranes. Differences within regions of the membrane itself also may be significant.

We have found some variation in the capacity of gelatin to induce large body formation; some lots were completely inhibitory. A subsequent paper will offer more data on the gelatin medium and the physiological conditions favoring growth of the L-form as a single large body.

The results of our studies constitute, we believe, the first demonstration of the viability of internal granules and contrast to those of Roux (14). Under the conditions of his experiments, granules were not demonstratively viable. He isolated agar-grown large bodies of only 5 to 6 μm in diameter, and if it follows that production of viable units is inefficient, then his chances of recovering CFU were probably very small. We were able to correlate the number of released CFU with the size of large bodies. Some bodies which were even three times the
diameter of Roux's isolates were shown to contain no CFU.

The lack of viability of the great majority of granules is likely to be related to the chance inclusion of a complete genome. Mechanisms of segregation of multiple chromosomes without mesosomal attachment and the incorporation of the genome into granules have not been established. Low levels of deoxyribonucleic acid have been detected in granules of less than 0.3 μm diameter (15), yet most such granules examined by transmission electron microscopy have been demonstrated to be empty vesicles or void of obvious threads of deoxyribonucleic acid (6, 8, 16).

Whether the intact large body can replicate is still questionable. Simple fission has not been observed, but we have noted forms which appear to be budding (3, 4); however, final separation was not seen. In hanging drops and occasionally in small amounts of broth single large bodies did not show any growth or replication. Conditions may not have been optimal but it is also possible that large bodies, so long as their outer membranes remain entire, may be end cells of growth. However, by mechanical trauma or by lysis of vacuoles from interior or exterior biochemical causes, viable internal units may be released from their confinement for further propagation. We have not observed the bursting of vacuoles by overfilling of granules. When rupture was rarely noted in agar colonies, it was spontaneous with release of a few granules (unpublished data). Bursting was not seen in broth cultures in exponential or early stationary phase, although it most likely occurs in death phase, for debris and fragmented large bodies can be found in old cultures.

Still unanswered is the relationship in size and nature of granules to their viability. Green et al. (10) have hypothesized that electron-dense bodies are "stem cells". Our data on via-

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**TABLE 1. Relation of number of viable internal granules to size of enclosing large body**

<table>
<thead>
<tr>
<th>Diam of large bodies (μm)</th>
<th>No. of samples</th>
<th>Internal CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–30</td>
<td>5</td>
<td>0–8</td>
</tr>
<tr>
<td>30–40</td>
<td>12</td>
<td>14–65</td>
</tr>
<tr>
<td>40–60</td>
<td>4</td>
<td>108–200</td>
</tr>
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Fig. 10. L-form of Streptococcus faecium strain F24 harvested from gelatin pour plate. Note that the form is a single, intact large body. Bar = 1 μm.
Fig. 11. Isolated large body of about 30-μm in diameter. Group A strain ADA-L. No exterior granules can be detected. ×1,100. High contrast film. (a) Arrow points to granule located between two vesicles. Plane of focus at bottom of body; (b) focus at middle; (c) focus at top; (d) large body being crushed between slide and cover glass. Arrow indicates probable site of membrane rupture and release of internal granules. Bar = 10 μm.
bility tends to fit the proportion of such granules to elementary bodies as seen from their micrographs. An understanding of the formation of such dense bodies will help solve this problem.

Green et al. (10) also believed that the collapsed vacuoles on the edge of large bodies were a result of bursting from the pressure of numerous internal granules. It is not clear why a membrane ruptured outward should necessarily collapse inward. We have observed such features in large bodies grown in our gelatin medium. We also have noted by SEM depres-
sions on the surface of large bodies in agar colonies (14). It is possible that once removed from the restraining gelatin matrix, granules were released. However, as almost all vacuoles in our preparations were vacant and those which did contain granules were hardly filled, the hypothesis of Green et al. (10) seems unlikely. As plausible, although morphological evidence is still deficient, is the concept of invasion in the formation of vacuoles. The reason for such surface depressions remains a puzzle.

ACKNOWLEDGMENTS

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