

RELEASE OF ENDOTOXIN IN THE FORM OF CELL WALL BLEBS DURING IN VITRO GROWTH OF *NEISSERIA MENINGITIDIS**

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The biochemically complex endotoxin makes up an integral part of the cell wall of Gram-negative bacteria (14). *Escherichia coli* incubated under conditions which inhibited protein synthesis (1, 17) released substantial amounts of this endotoxin into the surrounding medium in the form of cell wall blebs. These same cells, however, incubated under normal growth conditions released endotoxin in quantities so small as to make accurate measurement difficult (17). A similar release of lipopolysaccharide has been observed in *Pseudomonas aeruginosa* (11) and *Salmonella typhimurium* (12) grown under conditions of limiting phosphate. Electron microscopy studies on *Vibrio cholera* revealed surface blebs on log phase cells but not on cells in the stationary phase of growth (3).

Recent evidence in our laboratory from representative strains of three groups of meningococci indicates that a substantial amount of the outer cell wall layer—the layer proposed by several workers (5, 8, 9, 13) as the site of endotoxin—is released into the medium in the form of membranous blebs. In this paper we present an ultrastructural and biochemical study of the formation and subsequent release of endotoxin-containing cell wall bleb material from meningococci during normal in vitro growth.

Materials and Methods

Organism.—The strains of *Neisseria meningitidis* used in this study, group A(791), group B(SD1C), and group C(SD3C) were obtained from the *Neisseria* Repository, School of Public Health, Berkeley, Calif.

Maintenance of Stock Cultures.—Stock cultures in our laboratory were maintained in the lyophilized state. All cultures were reconstituted and examined for strain purity by procedures described by Vedros (19).

Cell Growth.—Cells from 24-h colonies on blood agar plates, previously inoculated with reconstituted lyophilized cells, were used as the inoculum for 10 ml of Bacto Mueller-Hinton broth (M-H broth) (Bacto Bacteriological Products, Difco Labs., Detroit, Mich.) which was incubated for 11 h (37°C) at 100 rpm on a rotor shaker. 0.1 ml of the 11-h culture was then used to inoculate 50 ml of fresh broth which was incubated 9 h (late log phase) at 37°C (100 rpm). For some experiments a portion of the 11-h culture was diluted in M-H broth and plated onto M-H agar plates. Plates were incubated in candle jars (100% humidity) at 37°C for 16 h.

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When appropriate, both broth and agar media were supplemented with 10% fresh normal bovine serum. In some experiments, 2.0 ml of an 11-h broth culture of SD1C strain were used as an inoculum for 1,000 ml of a defined broth medium consisting of Bacto Casamino acids without vitamins (0.1%), glucose (0.1%), KCl (0.01 M), MgSO₄ (2.5 mM), NH₄Cl (0.04 M), Tris (0.05 M), KH₂PO₄ (0.5 mM), and Fe(NH₄)₂SO₄ (0.02 mM), adjusted to pH 7.5. Cells were incubated with shaking at 37°C to mid-log phase (4 h). In some experiments, 10 ml of the above medium were made radioactive by the addition of D-[U-¹⁴C](2 μCi) glucose and protein [U-¹⁴C] hydrolysate (2 μCi) (both Amersham/Seale Corp., Arlington Heights, Ill.).

Viability Studies.—The viability of the meningococci was determined by colony counts on M-H agar plates inoculated with appropriate dilutions of cell suspension in M-H broth.

Direct Counts.—Direct counts on cell suspensions were determined with a Petroff-Hausser chamber (0.05 mm depth) using a Zeiss photomicroscope with phase optics (Carl Zeiss, Inc., New York).

Isolation of Cell Wall Blebs.—Whole cells and their attached blebs were removed from the growth medium by centrifugation (1500 g, 20 min) leaving cell-free blebs in the supernatant fluid. Whole cells were resuspended in fresh medium and subjected to 2 s of agitation in a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) to remove blebs attached to cell surfaces. This procedure was effective in removing blebs attached to cell surfaces but did not break whole cells. This procedure provided four fractions for extraction with aqueous phenol: whole cells, cell-free blebs from the growth medium, the culture supernatant fluid, and blebs removed from cell surfaces. Bleb fractions were collected by centrifugation (100,000 g, 1 h).

Extraction of Lipopolysaccharide (LPS).—LPS was extracted from each fraction with aqueous phenol (21). After a 4-day dialysis in the cold, extracts were subjected to treatment with DNase-RNase (50 μg/ml) at 37°C for 1 h. The LPS was further purified by repeated centrifugations (150,000 g, 3 h). The LPS samples were recovered as clear pellets.

KDO Determinations.—KDO (2-keto-3-deoxyoctonate) was determined in each fraction after hydrolysis of extracted LPS by the method of Weissbach and Hurwitz (20) and Osborn (15).

Radioactivity Determinations.—Samples were suspended in distilled water and added to appropriate volumes of PCS Solubilizer liquid scintillation fluor (Amersham/Seale Corp.). Radioactivity was determined by use of a Nuclear-Chicago Isocap 300 liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

Electron Microscopy.—Preparations of specimens for electron microscopy of thin sections was carried out essentially by the method of DeVoe et al. (6). Cells were prefixed by the addition of the appropriate amounts of glutaraldehyde to give a final concentration of 0.5% (pH 7.5). During prefixation the temperature of the cell suspension was slowly lowered to 4°C. Specimens were postfixated in 2.5% glutaraldehyde (1 h, 4°C) buffered with Veronal acetate. Further fixation was carried out in 1% OsO₄ (30 min) followed by treatment in 1% uranyl acetate in 30% ethanol (1 h). Specimens were dehydrated by passage through an ethanol series and embedded in Epon 812. Material was sectioned with an LKB Ultratome I (LKB Instruments, Inc., Rockville, Md.) and sections were double stained with uranyl acetate and lead citrate (16). All negatively stained samples were prepared with 1% phosphotungstate (pH 7.4).

All electron micrographs were taken with an AEI electron microscope 6B (AEI Scientific Apparatus Inc., Elmsford, N. Y.).

RESULTS

It is a general practice before bacteria are fixed and embedded for electron microscopy that they be harvested from the growth medium and washed by centrifugation with some suitable solution which will maintain their viability.

physiological activity, and ultrastructure. In our laboratory, meningococci treated in this manner exhibited cell structures in electron micrographs similar in most respects to those of other Gram-negative bacteria (Fig. 1) and identical to the normal meningococci observed by others (2, 18). The relatively electron-transparent nucleoid zone (n) contained fibrous material, presumably consisting of DNA. The area of the cytoplasm rich in ribosomes (r) was seen as an electron-dense zone enclosed in the cytoplasmic membrane (cm). The periplasmic space (pp), that space between the cytoplasmic membrane and the outer wall mem-

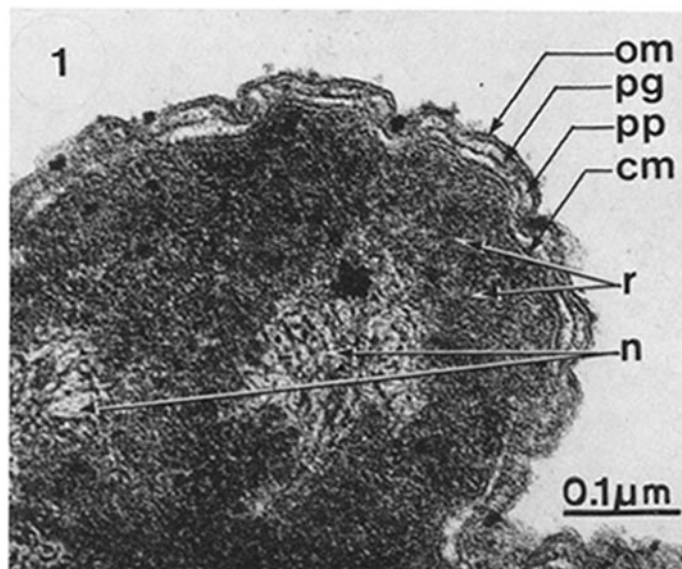


FIG. 1. Section of *N. meningitidis* SD1C (group B). Outer cell wall layer (om); peptidoglycan (pg); periplasm (pp); cytoplasmic membrane (cm); nuclear material (n); ribosomal area (r) $\times 170,000$.

brane (om), contained a densely stained layer (pg) in a matrix of a much less dense, amorphous material making up the periplasm. In the meningococcus, this very dense layer (pg) in the periplasm has been shown by Swanson and Goldschneider (18) to be sensitive to attack by lysozyme, an observation suggesting that this layer corresponds to the peptidoglycan component of the bacterial wall. The undulating outer membrane (om) appears also as a "unit membrane" measuring approximately 7.5 nm.

We have recently found that the meningococci fixed in the growth medium, without prior washing, exhibited multiple bleblike structures (Fig. 2 A-C) associated with the outer wall layer (om). In the group A (791) meningococcus the size of blebs was relatively constant measuring in the range of 30 nm while bleb size in group B (SD1C) cells varied from 40 to 75 nm with a mean size of about

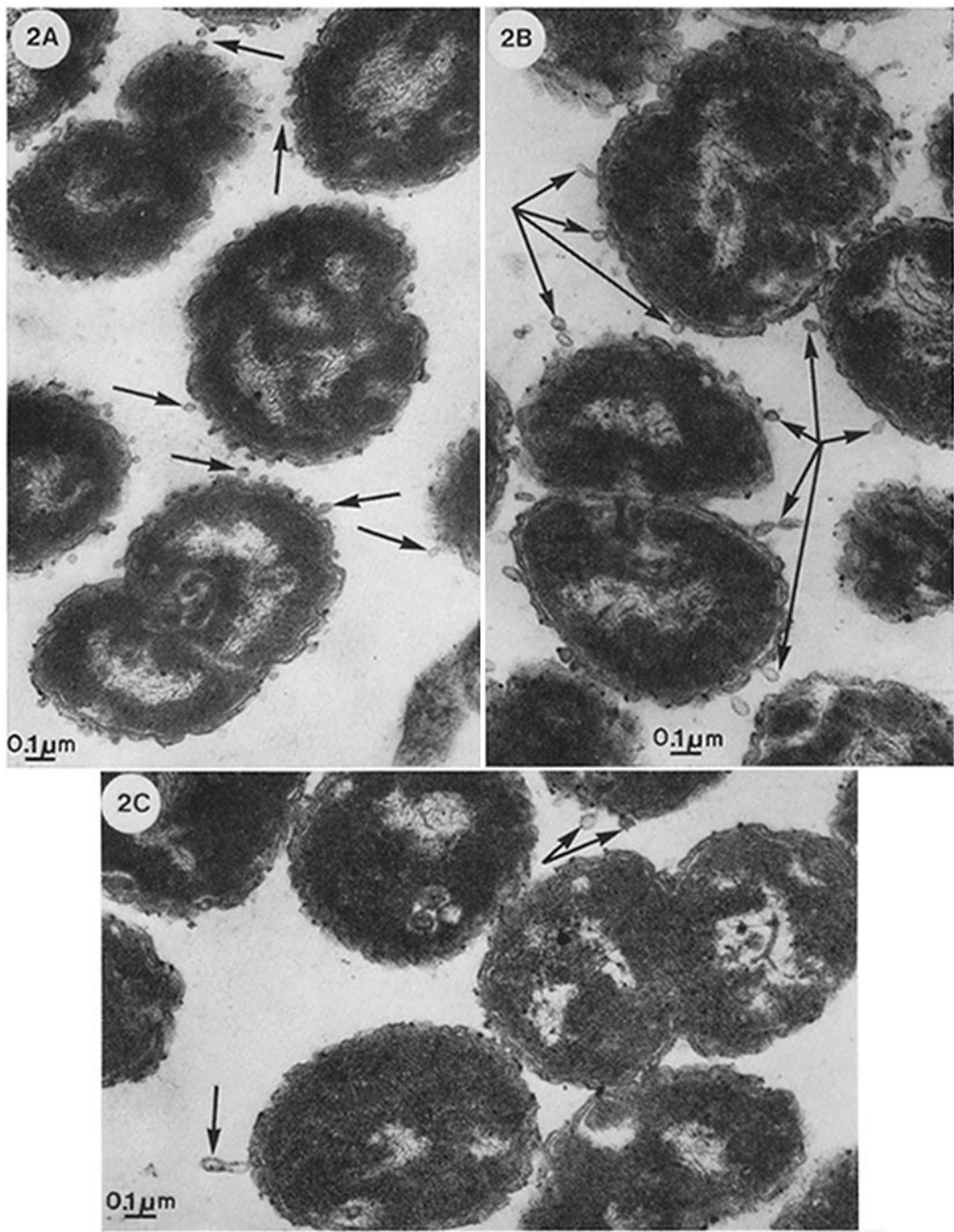


FIG. 2. Sections of three strains of meningococci fixed in the growth medium. $\times 45,000$. (A) Group A (strain 791); (B) group B (SD1C); (C) group C (SD3C). Large arrows point to cell wall blebs.

50 nm. Group C (SD3C) cells were conspicuous in that they exhibited relatively fewer blebs than did group A and B cells.

We considered the possibility that the blebs were merely membrane fragments from lysed cells which had attached to the surfaces of intact cells. Moreover, the blebs could have been an artifact due to the age of the culture, the nature of the medium (liquid or solid), or an environment lacking in serum components. Several experiments were carried out to test these possibilities.

To determine the origin of the blebs and to rule out artifacts due to glutaraldehyde fixation both fixed and unfixed cells from the growth medium were compared in negative stain. The results were identical from both preparations so only those of unfixed cells are presented (Fig. 3). The bleb material can be seen to originate (large arrows) as evaginations of the wall of the meningococcus. The presence of numerous cell-free blebs in these negative-stain preparations suggested that the blebs were given off into the growth medium as a normal conse-

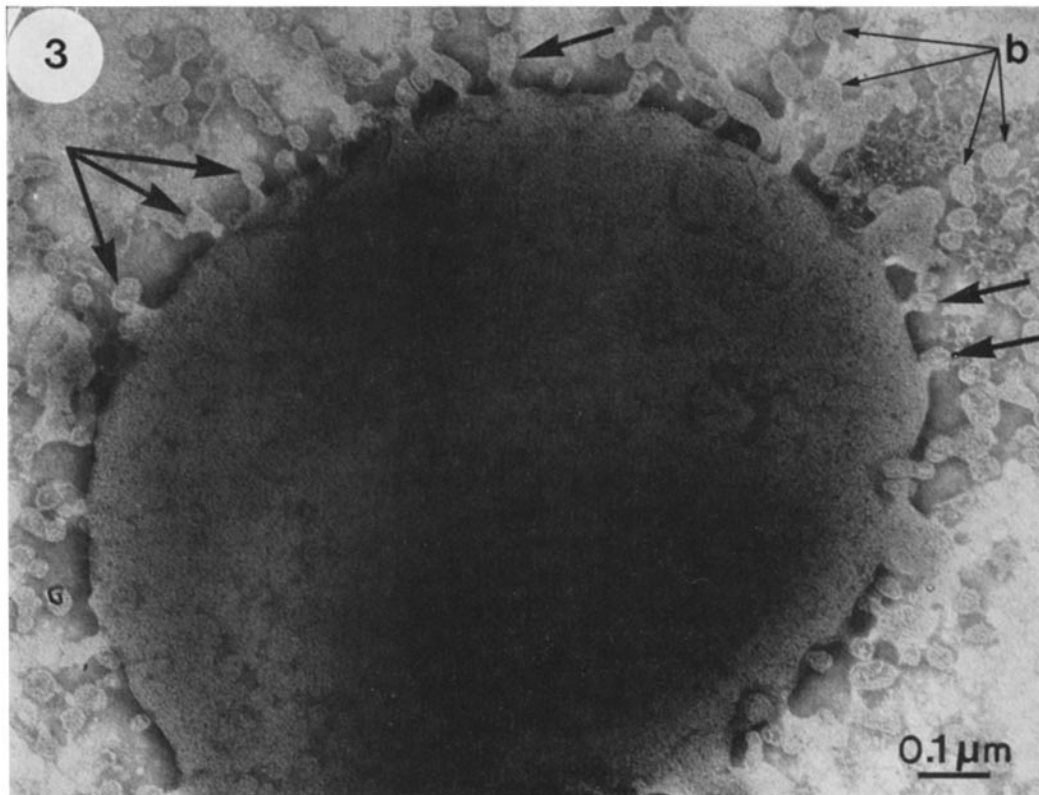


FIG. 3. *N. meningitidis* (SD1C), group B, from the growth medium. Large arrows point to evaginations of the cell wall (blebs) while small arrows (b) refer to extracellular blebs. $\times 95,000$.

quence of cell growth. Whole cells and cell-free blebs were easily separated by centrifugation. The culture supernatant fluids were then subjected to ultracentrifugation. Negative stains of the sedimented material from the culture supernatant fluids revealed pure preparations of bleb material (Fig. 4 A). From the group B cells the sedimented material frequently contained long tubular blebs (Fig. 4 B-D). Such tubular-type blebs were never observed on group A and C cells. The material inside the blebs is more dense than the surrounding embedding matrix (Fig. 4 D) indicating that periplasmic material was enclosed by the blebs. A schematic representation of bleb formation is presented in Fig. 5.

The age of actively growing cultures as a significant factor in the production of cell blebs was ruled out when log phase cultures from both solid and broth media were examined after 3, 6, and 9 h of growth. In any culture all the cells examined contain cell wall blebs; however, the majority of cells exhibited multiple surface blebs such as those shown in Fig. 3. The pattern was the same in all cultures examined, irrespective of culture age except that cells from stationary phase cultures (12 h) exhibited no surface blebs. The addition of 10% bovine serum to cultures, either broth or solid, had no visible effect whatever on the production of the blebs.

During this experimental work broth cultures were routinely examined for both viability and direct counts in order to determine relative numbers of viable cells in populations. The results ruled out the possibility of autolysis as a significant factor in bleb production. In each instance viable counts and direct counts were the same within the limits of experimental error ($\pm 5\%$).

Cell surface blebs were also observed when strain SD1C was grown in a glucose-amino acids medium (see Materials and Methods). To determine what proportion of the total cell material in a culture consisted of cell wall blebs, *N. meningitidis* group B (SD1C) cells were grown in a radioactive, amino acids-glucose medium. The flow diagram outlining the procedure used for the separation and isolation of ^{14}C -labeled whole cells and cell wall blebs is presented in Fig. 6. During this procedure "unbound" radioactive material was chased from both whole cells and blebs by washing those fractions in the growth medium containing unlabeled glucose and amino acids, a procedure applied successfully in earlier work with radiolabeled *Staphylococcus epidermidis* (7). The pattern for the removal of unbound label from whole cells and blebs was identical. The results shown in Fig. 7 are those from the whole cells. An analysis of the relative quantities of radioactivity bound by the various fractions (Table I) indicates that the bleb fraction has retained 11% of the total radioactivity. As previously mentioned, negatively stained samples of the pellet from high speed centrifugations consisted of pure bleb material. Therefore, the findings from the radioactive studies indicate that these cell wall blebs represent a substantial proportion of the cell material in the culture.

The relative content of endotoxin in aqueous-phenol extracts of bleb fractions and whole cells was determined by analyses for KDO, a unique component

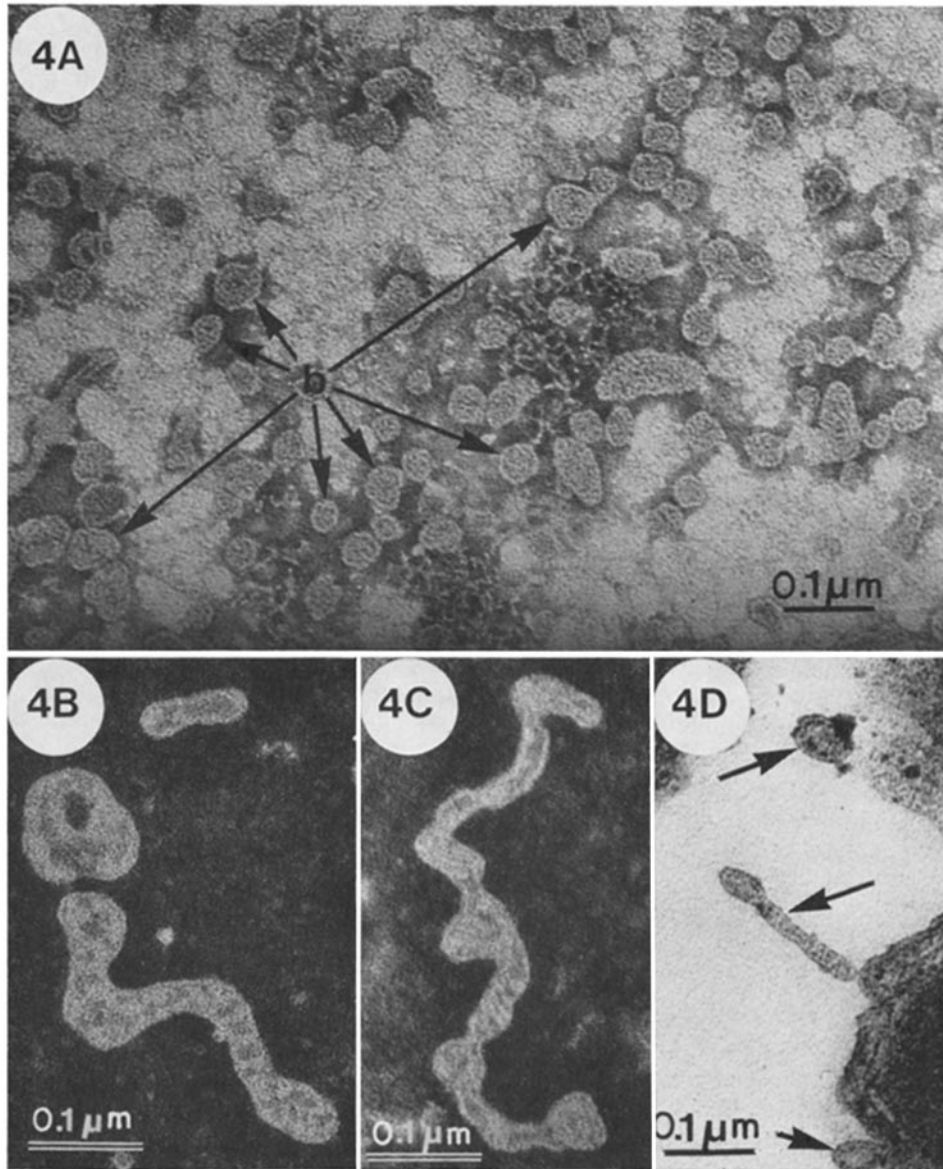


FIG. 4. (A) Negative stain of cell wall blebs (*b*) isolated from the culture supernatant fluid by ultracentrifugation. (B) and (C) Negative stains of frequently encountered tubular-type bleb from group B (SD1C). (D) Section of tubular and spherical blebs from group B (SD1C) cells. Magnifications: A, $\times 120,000$; B, $\times 150,000$; C, $\times 150,000$; D, $\times 120,000$.

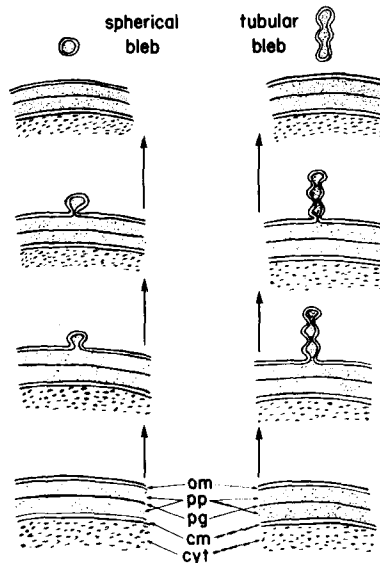


FIG. 5. A schematic representation of the formation and release of surface blebs from *N. meningitidis*. This diagram is a composite of many observations from electron micrographs taken throughout this study. Cytoplasm (*cyt*). All other labels are as given in legend of Fig. 1.

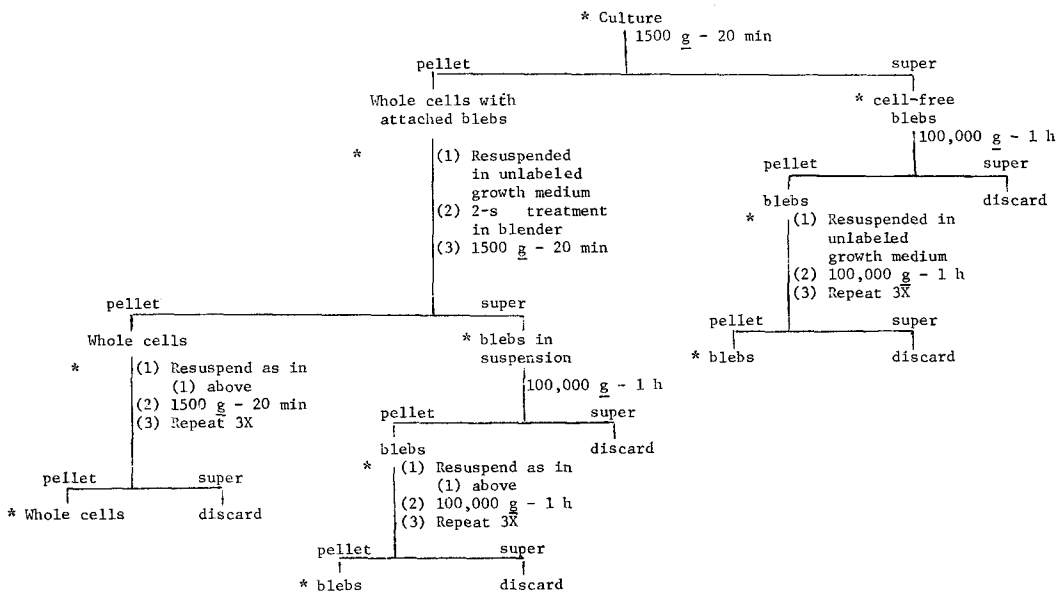


FIG. 6. Flow diagram for separation of whole cells, cell-associated blebs, and cell-free blebs. Points at which samples were removed for radioactive determinations (*). The volume of all suspensions was identical to that of the growth medium (20 ml).

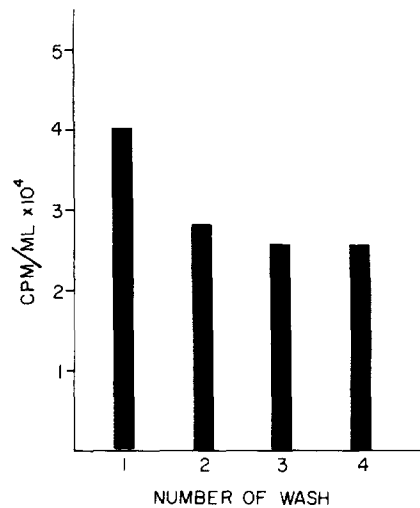


FIG. 7. Removal of unbound ^{14}C -labeled material by repeated washes of cells in nonradioactive growth medium.

TABLE I
*Relative Distribution of ^{14}C Label between Whole Cells, Cell-Associated Blebs, and Cell-Free Blebs in Broth Culture of *N. meningitidis* Group B (SD1C)*

	Bound ^{14}C	Radioactivity
	<i>cpm/ml</i> *	%
Whole cells	25,820	89
Cell-associated blebs	803	3
Cell-free blebs	2,206	8
Total	28,829	100

* The volumes of all suspensions were identical to that of the growth medium.

of the LPS (endotoxin) of the meningococci (10) as well as other Gram-negative bacteria (14). The results of these analyses (Table II) indicate that 18% of the total KDO of the culture was present as cell wall blebs. Under the conditions tested this release of relatively large quantities of endotoxin-containing material into the culture supernatant fluid appears to be a consequence of normal in vitro growth of the SD1C strain of meningococcus examined here.

DISCUSSION

We have presented evidence here for the formation and release of large numbers of cell wall blebs during the normal log phase growth of three strains of *N. meningitidis*. Such blebs were not associated with cell lysis nor with the age of

TABLE II
*Relative Levels of KDO in Aqueous Phenol Extracts of Whole Cells, Cell-Associated Blebs, Culture Supernatant Fluid, and Cell-Free Blebs**

Sample	KDO	
	Micromoles	Relative %
Whole cells less surface blebs	68	72
Cell-associated blebs	5	6
Cell-free blebs	10	12
Culture supernatant fluid‡	0	0
Total	83	100

* Figures are based on a 1-liter culture in Casamino acids medium (see Materials and Methods).

‡ Below limits of detection.

the culture during log phase growth; however, cells in the stationary phase of growth did not exhibit surface blebs in our negative-stain preparations. Cells from both broth and solid media, with or without serum, exhibited large numbers of surface blebs. These observations suggest that release of cell wall material in these meningococcal strains may be a general characteristic of the meningococcal growth pattern. A similar phenomenon of bleb formation was observed by Chatterjee and Das (3) during the in vitro growth of a strain of *V. cholerae*. These authors proposed a possible relationship between such surface structures and the pathogenicity of *V. cholerae*.

It is a commonly held conception that pathogenic Gram-negative bacteria must lyse or be mechanically disrupted to release significant amounts of their endotoxin (4). Liberation of large amounts of endotoxin (LPS) from viable *E. coli* has been reported; however, such cells were held under conditions which inhibited protein synthesis and, therefore, cell division (1, 17). Both *S. typhimurium* (12) and *P. aeruginosa* have been reported to excrete LPS under conditions of phosphate limitation (11). The release of endotoxin-containing cell wall blebs from the meningococci during our experiments occurred under conditions of growth without apparent limiting nutrients.

The biochemical and physiological properties of the blebs released from *N. meningitidis* are presently under investigation in this laboratory. It is already apparent from these studies that the cell-free blebs contain endotoxin (Table II). The origin of these blebs at the outer membrane layer of the cell wall is particularly significant, as it is this layer which has been designated in several Gram-negative bacteria as the site of endotoxin in the cell (5, 8, 9, 13). It is interesting to speculate that a mechanism such as we have described here for cells grown in vitro may also operate in vivo. Were this the case, such a mechanism could account for the release of substantial amounts of cell-free endotoxin during active, uninhibited growth of the organism in vivo.

SUMMARY

Multiple cell wall blebs were observed on the surface of three strains of *N. meningitidis* taken from log phase cultures. The blebs originated as evaginations of the outer layer of the cell wall. Bleb production was noted on both defined or complex media either as broth or a solid medium. The addition of 10% normal bovine serum to the various media did not affect the production and release of these surface blebs. However, as broth cultures progressed into the stationary phase of growth, the blebs disappeared from the surface of the cells. Blebs were present in substantial quantities in culture supernatant fluids and on cell surfaces and were readily isolated by ultracentrifugation. Analysis for 2-keto-3-deoxyoctonate in cultures revealed that 18% of the total endotoxin of log phase cultures was present in blebs from the cell wall.

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