The effect of *Pseudomonas aeruginosa* cytotoxin and toxin A on human polymorphonuclear leukocytes

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Summary. After exposure to cytotoxin or toxin A of *Pseudomonas aeruginosa*, the ultrastructure of resting and phagocytosing human polymorphonuclear leukocytes (PMNL) and of cells of *P. aeruginosa* strain 1348A was studied by transmission (TEM) and scanning (SEM) electronmicroscopy, and by light microscopy (LM) after histochemical staining of cytoplasmic granules. Cytotoxin caused marked clumping and destruction of PMNL, pyknotic nuclear changes with bleb formation, and release of cytoplasmic granules; phagocytosis was markedly diminished. In contrast, after exposure to toxin A, PMNL phagocytosed actively, but their cytoplasmic pseudopodia were markedly irregular and their nuclei pyknotic. Colloidal-gold-labelled cytotoxin showed an affinity for the cytoplasmic membranes, nuclei and granules of PMNL. Cytotoxin had no apparent effect on cells of *P. aeruginosa* strain 1348A but there was polar separation of the cytoplasmic membrane in bacteria exposed to toxin A. Cytotoxin and toxin A appear to be important in the pathogenesis of infections caused by *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa produces several substances that may influence the severity of the infections it causes (Liu *et al.*, 1961; Liu and Hsieh, 1973; Baltch *et al.*, 1979; Young, 1980; Pavlovskis and Wretling, 1983; Middlebrook and Dorland, 1984). For example, toxin A inhibits mammalian-cell protein synthesis by inactivation of elongation factor 2 (Iglewski and Kabat, 1975) and toxin A at concentrations as low as 30 ng/ml caused morphological changes in human macrophage tissue cultures (Pollack and Anderson, 1978). Both of these effects were abolished by specific antibody. Cytotoxin (formerly called leukocidin) is toxic to all eukaryotic cells except the thrombocytes and erythrocytes of some mammalian species (Scharmann, 1976*a, b, c* and *d*; Scharmann *et al.*, 1976; Lutz, 1979; Baltch *et al.*, 1985). In earlier studies, cytotoxin decreased the function of human polymorphonuclear leukocytes (PMNL) and subsequently destroyed them. Although cytotoxin appears to act primarily on the cell membrane, the incorporation of tritiated thymidine into DNA is decreased in murine

splenocytes exposed to cytotoxin (Obrig *et al.*, 1984). This effect appears to be intimately related to Ca^{2+} -dependent systems of the cell (Scharmann *et al.*, 1976).

This study describes some of the effects of cytotoxin and toxin A from *P. aeruginosa* on the ultrastructure of human PMNL and on cells of *P. aeruginosa* strain 1348A, alone or interacting during phagocytosis, observed by transmission (TEM) and scanning (SEM) electronmicroscopy. Observations on the histochemical staining of PMNL exposed to either toxin, and studies with cytotoxin labelled with colloidal gold, are included.

Materials and methods

Preparation of PMNL

Serum and PMNL were obtained from healthy hospital personnel. PMNL separated by dextran sedimentation were suspended in Eagle's Minimal Essential Medium (MEM) with Earle's salts and L-glutamine, and centrifuged at room temperature for 30 min at 400g through lymphocyte-separation medium (Litton Bionetics, Kensington, MD, USA). After hypotonic lysis of erythrocytes, PMNL were washed with MEM and resuspended at a concentration of $2.5 \times 10^6/\text{ml}$ in Hanks's Balanced Salts Solution-gel (HBSS-g) containing pooled normal human serum 20%. This suspension was divided into four aliquots for study: (a) as a control, PMNL not treated

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further; (b) as a control, PMNL rotated end-over-end for 2 h at 37°C; (c) PMNL exposed to toxin A at a concentration of 1250 ng/ml during end-over-end rotation for 2 h at 37°C; (d) PMNL exposed to cytotoxin at a concentration of 4 µg/ml during end-over-end rotation for 2 h at 37°C.

P. aeruginosa preparation

Cells of *P. aeruginosa* strain 1348A (obtained from a patient with bacteraemia) were grown on Mueller-Hinton Agar for 18 h at 37°C, washed twice in Hanks's Balanced Salts Solution (HBSS) and resuspended in cold HBSS until use (Hammer *et al.*, 1981).

PMNL phagocytosis studies

Control PMNL and PMNL exposed to cytotoxin or toxin A, as described above, were mixed with cells of *P. aeruginosa* strain 1348A in the ratio PMNL:bacteria, 1:20, rotated end-over-end (Hammer *et al.*, 1981) for 1 h at 37°C, then prepared for ultrastructure studies.

P. aeruginosa toxins

Cytotoxin was purified as described by Lutz (1979). Toxin A was purified as described by Taylor and Pollack (1978).

Ultrastructure studies (Hayat, 1977)

Transmission electron microscopy (TEM). PMNL for TEM were centrifuged at 100 *g* for 5 min in a Wintrobe tube. The supernate was replaced with glutaraldehyde 1% w/v in 0.1M sodium cacodylate buffer, pH 7.4. The cell pellet was fixed overnight at 4°C, washed in 0.1M sodium cacodylate buffer, post-fixed with osmium tetroxide 1% w/v (buffered to pH 7.4 with 0.1M sodium cacodylate buffer) for 1 h, then dehydrated in a graded series of ethanols, followed by propylene oxide, and embedded at 70°C overnight in Polybed 812 (Polysciences, Inc., Warrington, PA, USA).

Sections 50–70 nm thick, cut on a LKB ultramicrotome with a diamond knife (E. I. duPont de Nemours & Co., Inc., Wilmington, DE, USA) were placed on 300-mesh copper grids (Ted Pella Inc., Tustin, CA, USA) and, to enhance contrast, were stained for 15 min each with uranyl acetate in methanol and with lead citrate. The sections were examined and photographed in a Philips 300 transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ, USA).

Scanning electron microscopy (SEM). For SEM, PMNL were prepared as for TEM. The PMNL suspension was fixed for 30 min in glutaraldehyde 1% w/v in 0.1M sodium cacodylate buffer, pH 7.4, then transferred to acetone-cleaned aluminium stubs (Electron Microscopy Sciences, Fort Washington, PA, USA) to which fresh glutaraldehyde 1% was added. During all phases of processing the cells were kept covered by fluid to avoid drying. After fixation overnight at 4°C, the cells were rinsed in

cacodylate buffer, dehydrated in a graded series of ethanols, dried in a critical point dryer (Tousimis Research Corp., Rockville, MD, USA) and coated with a fine layer of gold in a vacuum evaporator (Kinney Vacuum Company, Boston, MA). The cells were examined and photographed on an ISI 60A Scanning Electron Microscope (International Scientific Instruments Inc., Santa Clara, CA, USA) with Type 52 Polaroid film (Polaroid Corporation, Cambridge, MA, USA).

Histochemical staining. Duplicate smears of the PMNL suspension were stained for leukocyte alkaline phosphatase (LAP) or combined AS-D chloracetate esterase and a naphthyl acetate esterase (dual esterase) to examine the granule population in whole cells and the cell morphology by light microscopy, and to ensure that groups of cells selected for ultrastructural study represented the preparation as a whole. Commercially available staining kits (Sigma Chemical Co., St Louis, MO, USA) were used; fixation, substrate application and counter-staining were performed according to the manufacturer's recommendations. The stained preparations were examined and photographed in a Wild M-20 light microscope (Upstate Technical Equipment Co., Inc., Syracuse, NY, USA).

Colloidal gold labelling (Horisberger and Rosset, 1977; Schwab and Thoenen, 1978; Bendayan, 1984). Sections 50–70 nm thick of fixed, embedded, non-rotated PMNL were placed on 200-mesh nickel grids (Ted Pella Inc.) and etched by floating the grid, with the sections lowermost, on a drop of hydrogen peroxide 10% for 15 min. After washing thrice with 0.01M phosphate buffered saline (PBS), pH 7.4, the sections were floated for 30 min on a drop of horseradish peroxidase (HRP) (Sigma) 1 mg/ml in 0.01M PBS to block non-specific background staining. After three further washings in 0.01M PBS, the sections were incubated with cytotoxin bound to HRP and colloidal gold particles (CGP) (E-Y Laboratories, Inc., San Mateo, CA, USA) diluted 1 in 4 to 1 in 6 in 0.01M PBS by adding a large drop of each solution to the sections on the grid and incubating for 1.5 h at room temperature. The CGP were of 10-nm particle size, contained HAuCl₄ 0.1% w/v, and had an absorbance peak at 510 nm; the protein concentration of the cytotoxin-HRP-CGP preparation was 50 µg/ml and of the HRP-CGP preparations 20 µg/ml. The sections were washed by agitation in 0.01M PBS for 10 min, washed thrice in distilled water and dehydrated for 1 min in each of a graded series of ethanols. Unstained sections and sections stained for 1 min with uranyl acetate 1% w/v in methanol were examined in a Philips 300 transmission electron microscope.

Results

Smears of each cell suspension were examined to determine the number and distribution of granules in whole cells and to ensure that groups of cells selected for ultrastructural study were representative. When stained for LAP and for chloracetate and non-specific esterases, primary, secondary and

tertiary granules of PMNL and monocyte granules were seen (Young and Prager, 1962; Rustin *et al.*, 1979). The distribution and pattern of granules was the same with either stain, but the LAP stain proved more suitable for this study because of better granule definition and because the PMNL examined consisted predominantly of cells in a late stage of development in which tertiary granules were present in increased numbers.

Control PMNL preparations

When examined by LM and TEM, untreated, unrotated PMNL were well preserved, separate, and had short blunt villous processes uniformly distributed at the cytoplasmic membrane. The

nuclei were normal and segmented with peripheral chromatin condensation. The cytoplasm was fairly uniformly and moderately electron dense, and contained evenly dispersed ribosomes. The specific granules, while pleomorphic and differing in electron density, were uniformly distributed throughout the cytoplasm. Mitochondria were small and the Golgi areas and cytoplasmic tubules and filaments inconspicuous (fig. 1b). There was little cell debris in the preparation (fig. 1). When examined by SEM, similar rounded cells with uniformly distributed, short villous process were seen (fig. 1c). Histochemical staining showed well-defined cytoplasmic granules (fig. 1a).

Control PMNL rotated for 2 h were morphologically different. When examined by LM most of the

Fig. 1. Control PMNL. (a) LAP stained smear; (b) TEM; (c) SEM. Resting PMNL have short, blunt, uniformly distributed villous processes. Granules are evenly distributed in the cytoplasm.

cells were spherical, but slightly smaller than unrotated cells. Their granules were slightly larger, fewer in number, but fairly uniform in distribution (fig. 2a). When examined by TEM, most of the cells were spherical, but showed some peripheral clearing of the cytoplasm with a few irregularly distributed, broad-based pseudopodia and bleb-like protrusions of the cytoplasm (fig. 2b). Both LM and TEM revealed a few cells with elongated cellular protrusions of this type which appeared to be separating at their bases by a process resembling clasmatosis. Free fragments, presumably shed in this way, were present between the PMNL, together with fragments of cell membranes. When examined by SEM, these changes were seen in three dimen-

sions; the preparations contained generally rounded cells showing peripheral flattening and irregularly scattered blebs upon the surface of an otherwise rather smooth cell membrane (fig. 2c).

Effect of toxin A on PMNL

When examined by LM, PMNL exposed to toxin A 1250 ng/ml were generally similar to those in the control preparation except that most had jagged, irregular extensions of their cytoplasm (fig. 3a). When examined by TEM, their electron density varied markedly. Some had a rounded profile and a few cellular cytoplasmic extensions containing many granules. The less electron-dense cells showed

Fig. 2. Control PMNL after end-over-end rotation for 2 h. (a) LAP stained smear; (b) TEM; (c) SEM. The cytoplasmic membrane is smooth except for broad-based blebs and pseudopodia (arrows) whose cytoplasm is cleared of granules.

less regularly distributed granules and the greatest cell-membrane activity. A majority of PMNL showed some rounding of their nuclear segments and early pyknotic changes; there were also many cell fragments between the cells (fig. 3b). Cells examined by SEM were similar to controls with few showing irregular projections among the more rounded cells (fig. 3c). Histochemical staining showed well defined intra-cytoplasmic granules.

Effect of cytotoxin on PMNL

Earlier studies on the effects of cytotoxin on PMNL (Baltch *et al.*, 1985) showed that the cytotoxin effects were time and dose dependent. Cytotoxin at concentrations $< 2 \mu\text{g/ml}$ had little effect on the structure of PMNL examined by TEM and $10 \mu\text{g/ml}$ produced complete cell lysis. In the

present study an intermediate concentration ($4 \mu\text{g/ml}$) was used to observe cell damage. When examined by LM, some areas of smears of PMNL exposed to cytotoxin at a concentration of $4 \mu\text{g/ml}$ showed round separate PMNL with as many granules as controls, but showing nuclear pyknosis. In other areas there were clumps of cells at an advanced stage of lysis, releasing granules (fig. 4a). When examined by TEM, the cell clumps consisted mainly of fragments of severely damaged cells with indistinct outlines, a few scattered granules and poorly defined cell organelles. Few cells had intact membranes; most were pleomorphic with a variable number of granules and pale, flocculent cytoplasm, and many had irregular cytoplasmic extensions free of granules. Most cells exhibited occasional lipid vacuoles and their nuclei showed pocketing and pyknosis (fig. 4b, d). When examined by SEM, the

Fig. 3. PMNL exposed to toxin A 1250 ng/ml after rotation for 2 h. (a) LAP stained smear; (b) TEM; (c) SEM. Most PMNL are rounded, but a significant number of them show jagged irregular cytoplasmic extensions (arrows) and irregularly distributed granules.

clumps consisted of collapsed PMNL with coalescent cell membranes surrounded by cell debris (fig. 4c). Histochemical staining showed granules mainly outside the PMNL cell membranes.

Phagocytosis in the presence of toxin A or cytotoxin

When examined by TEM, bacteria of strain 1348A added to control PMNL and rotated for 1 h were seen within viable PMNL in single or coalescent vacuoles and at various stages of disso-

lution (fig. 5a). The PMNL contained fewer granules than the controls; they were irregularly distributed in the cytoplasm. Some PMNL had ingested cytoplasmic fragments. However, even after this prolonged handling, the phagocytic function of the control PMNL appeared unimpaired.

When bacilli of strain 1348A were added to PMNL treated beforehand with cytotoxin, only a few scattered intact PMNL ingested bacteria and there was little evidence of lysosomal digestion (fig.

Fig. 4. PMNL exposed to cytotoxin 4 μ g/ml after rotation for 2 h. (a) LAP stained smear showing a cluster of cells at an advanced stage of lysis with release of granules; (b) and (d) TEM of similar areas: In (b) a few platelets (p) and red cells (rbc) remain among PMNL ghosts and cell debris; in (d) a few residual intact cells show a nuclear pocket (arrow) and nucleus (N) undergoing karyolysis (c) SEM showing a clump of collapsed PMNL with coalescing membranes.

Fig. 5. PMNL phagocytosis. (a) Control; bacteria (arrows) are seen within PMNL in single or coalescent vacuoles. (b) PMNL treated with toxin A are similar to the control. (c and d) PMNL treated with cytotoxin; (c) the few intact PMNL contain one or two phagocytosed bacteria, and (d) most bacteria uningested by PMNL, lie free in the cell debris.

5c, d). In contrast, PMNL treated with toxin A had the same unimpaired phagocytic killing capacity as controls (fig. 5b). Examination of groups of PMNL in more than 20 micrographs did show, however, that the PMNL vacuoles were slightly larger and that there was more swelling and disruption of bacteria than in control preparations.

Effect of the toxins on P. aeruginosa

When examined by TEM, cells of strain 1348A incubated with cytotoxin at a concentration of 4 $\mu\text{g/ml}$ were not demonstrably different from controls (fig. 6b). In contrast, bacteria incubated with toxin A at a concentration of 1250 $\mu\text{g/ml}$ were swollen and more of them were degenerated, many having a wrinkled outer membrane and showing polar separation of the cytoplasmic membrane from the cell wall (fig. 6a).

Localisation of colloidal gold-labelled cytotoxin

When PMNL were exposed to electron-dense probes, i.e., either to a complex of colloidal gold and cytotoxin (CGP-PAC) or, as a control, to HRP-CGP, and examined by TEM, the latter failed to take up the gold label (fig. 7a), while in the former irregularly spaced clusters of the gold label were

seen on the outer cell membrane (fig. 7b), and there was consistent but less marked labelling of the nuclei and granules.

Discussion

This study has shown that both cytotoxin and toxin A of *P. aeruginosa* cause changes in the ultrastructure of PMNL exposed to them. It is known that low concentrations of cytotoxin, i.e., 0.1 $\mu\text{g/ml}$, by increasing the permeability of PMNL membranes (Frimmer *et al.*, 1976, Lutz *et al.*, 1982) can allow small molecular weight substances, e.g., ruthenium red, to enter the cytoplasm (Baltch *et al.*, 1985). At the higher concentrations of cytotoxin, i.e., 4 $\mu\text{g/ml}$, used in this study, cytotoxin affected PMNL by causing cell fragmentation, release of cytoplasm granules, and decreased function. The few intact cells that survived its action showed nuclear pyknosis and pocketing. This latter phenomenon, which is believed to be due to an anomaly of mitosis during maturation, is often seen in leukaemic cells and in cells exposed to nitrogen mustard or ionising radiation (Ahearn *et al.*, 1967; McDuffie, 1967, Duplan *et al.*, 1969) and suggests that cytotoxin affects not only the cell membrane but the nucleus as well. The fact that cytotoxin labelled with colloidal gold attached to PMNL

Fig. 6. Effect of cytotoxin and toxin A on *P. aeruginosa*. (a) *P. aeruginosa* incubated with toxin A (TEM); there is prominent polar separation of the outer membrane (arrow) (b) *P. aeruginosa* incubated with cytotoxin showing unaffected bacteria (TEM).

Fig. 7. Colloidal gold labelling of PMNL. (a) Control PMNL with no binding of the label. (b) The colloidal gold-cytotoxin complex shows clusters of label (arrows) on the cell membrane surface, on PMNL granules (g) and on the nucleus (N).

granules, nuclei and cell membrane, supports the view that the action of cytotoxin is not restricted solely to the cell membrane. The release from PMNL of cytoplasmic granules, demonstrated by histochemical staining, suggests that the inflammation accompanying infection caused by *P. aeruginosa* may be due, at least in part, to PMNL lysosomal enzymes released by the action of cytotoxin.

In contrast, toxin A did not affect adversely either the morphology or the phagocytic ability of PMNL. However, examination by TEM demonstrated clearly changes representing cell damage in the form of jagged protrusions from the cell membrane, flocculent change in the cytosol and early pyknotic nuclear changes. These observations may be analogous to those seen with staphylococcal

α toxin (Gemmell *et al.*, 1982), i.e., enhanced phagocytosis was observed in the presence of low concentrations of toxin whereas higher concentrations reduced PMNL function.

Although cytotoxin did not affect the morphology of *P. aeruginosa* itself, toxin A caused wrinkling of the cell wall and separation of the cytoplasmic membrane from the cell wall at one pole. The significance of this observation is uncertain and requires further study.

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