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Application of Flow Cytometry to Monitoring of Liposomal Restructuring Induced by *Listeria monocytogenes*

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Abstract Liposomal restructuring induced by hemolytic *Listeria monocytogenes* was investigated by using flow cytometry. When added to calcein-entrapped liposomes, hemolytic, but not non-hemolytic, *Listeria monocytogenes* were able to induce reformation of vesicles. Such restructuring of liposomes was easily monitored by flow cytometry. Electron microscopy also indicated major changes in the challenged liposomal structures. The preliminary results described may offer a simple and fast method for monitoring liposomal restructuring and for differentiating between hemolytic and non-hemolytic bacteria.

Key words: *Listeria monocytogenes*, liposome, flow cytometry, phospholipases

Pathogenic *Listeria monocytogenes* is a Gram-positive bacterium that causes serious infection in humans [2, 12, 13]. Pathogenic, but not nonpathogenic, *Listeria* produces several extracellular products, including phospholipase C (PLC) and listeriolysin O [3, 13]. These enzymes are associated with specific catalytic biochemical transformations of mammalian cell membranes [5, 6, 9]. Therefore, the hemolytic action of *L. monocytogenes* is the main factor which distinguishes it from other nonpathogenic listeriae [3]. Apart from studies of the actions of hemolytic agents on intact cell membranes, the leakage of intracellular materials by bacterial hemolytic factors was investigated by several workers by using liposomes as an artificial model membrane [7, 8, 16]. This study primarily seeks to clarify the mechanisms of liposomal restructuring in the presence of hemolytic and non-hemolytic bacteria by using calcein-entrapped liposomes and flow cytometry. In addition, a preliminary analytical study of the hemolytic actions of bacterial enzymes on liposomes was also undertaken, using transmission electron microscopy (TEM).

Listeria monocytogenes NCTC 7973 hemolytic and non-hemolytic derivatives [13] and *Escherichia coli* NCIB 10772 (non-hemolytic) were obtained from the Department of Life Sciences, King's College London. The hemolytic activity of each strain was checked by subculturing the bacteria on horse blood agar plate (Unipath). Approximately 50 ml of sterile BHI broth (Oxoid) was inoculated with 0.5 ml of an overnight grown bacterial culture and incubated aerobically in a shaking incubator (200 rev min^{-1}) at 37°C. Early stationary phase bacteria were harvested by centrifugation (10,000 ×*g*, 15 min, 4°C) and washed three times in a buffer (0.01 M sodium phosphate buffer, pH 7.0). The washed bacteria were resuspended in the buffer to the desired concentration of bacteria, estimated from a preestablished bacterial concentration calibration curve (colony forming unit, cfu ml^{-1} *vs.* O.D. at 600 nm) for each strain.

Fluorescent dye-entrapped small unilamella vesicles (SUVs) were prepared with commercially available pure phosphatidylcholine [(PC), from egg yolk, phosphatidylcholine content 99%: Sigma] and calcein (Sigma). The PC solution was dried in a vacuum evaporator for 1 h at 60 rpm at room temperature. After all the solvent was removed, the PC film was hydrated, using 0.08 M calcein-buffer (0.01 M Na-phosphate, pH 7.0) solution. SUVs were prepared by sonication of PC-calcein suspension with a sonicator (Branson, Model 250; power setting: 5; duty cycle: 30%, 12 min). The emulsion was then passed through a Sephadex (G-50, Sigma) column to purify fractions containing calcein-entrapped liposomes [7, 15].

A flow cytometric method was used to analyze the size of liposomes treated with hemolytic bacteria or bacterial culture supernatant. Bacterial suspension (0.2 ml of 5.0×10^{8} cfu ml^{-1}) was added to freshly made calcein-entrapped liposome suspension (0.1 ml) which was diluted in 1.9 ml

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of Na-phosphate buffer. In the case of control experiments, 0.2 ml of buffer was added to the diluted liposome suspension, instead of bacterial suspension. The bacteria and liposomes were shaken, using the reciprocating shaker, for 30 min (200 rev. min^{-1}) at 30°C. The mixture was then centrifuged (3,000 $\times g$ for 10 min), and the liposome-containing supernatant was collected. A bench top flow cytometer (FACSCAN, Becton Dickinson) was used to analyze the size of liposomes, using light-scattering and fluorescence measurements. Samples were illuminated at 488 nm by an argon ion laser, and fluorescence emission was detected at 520 to 550 nm.

For direct size analysis, liposomal calcein and other treated liposomes were examined by transmission electron microscopy (JEOL, Model 100 CX-2 Transmission Electron Microscopy, Japan). For negative staining, 1% ammonium molybdate in double distilled water was used [15].

The reactions between liposomes and various bacteria strains were carried out as previously described. After completion of the reaction, the changes in fluorescence intensity and size of liposomes from each liposome-containing reactant sample was examined by a flow cytometer [4].

Figure 1 depicts overlays of fluorescence histograms from the reaction of bacteria with liposomes. These results show that the liposomes that were challenged by hemolytic *L. monocytogenes* showed a much higher fluorescence intensity (Fig. 1A) than those treated by non-hemolytic *Listeria* and *E. coli* or untreated liposomes (1B, 1C, and 1D in Fig. 1, respectively). When calcein is in the liposomes at a high concentration, it has a relatively low fluorescence intensity but, if diluted, the fluorescence

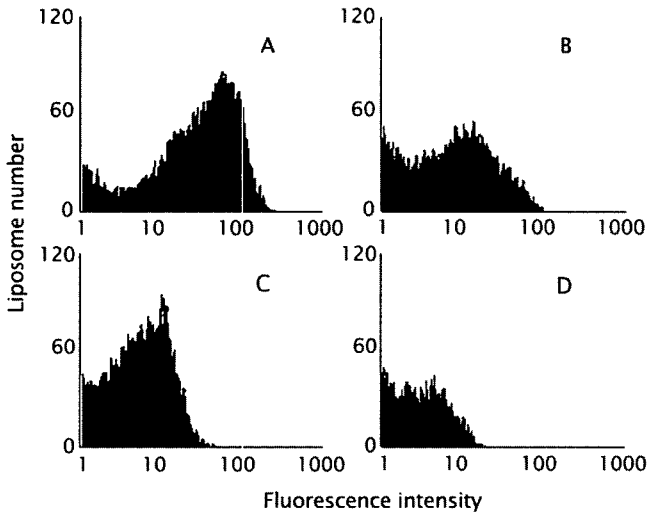

Fig. 1. Change of fluorescence intensity of calcein, examined by flow cytometry, following the reaction of liposomes with bacteria.
(A) *L. monocytogenes* (NCTC 7973, hemolytic strain); (B) *L. monocytogenes* (NCTC 7973, non-hemolytic strain); (C) *E. coli* NCIB 10772; (D) control (untreated liposomes).

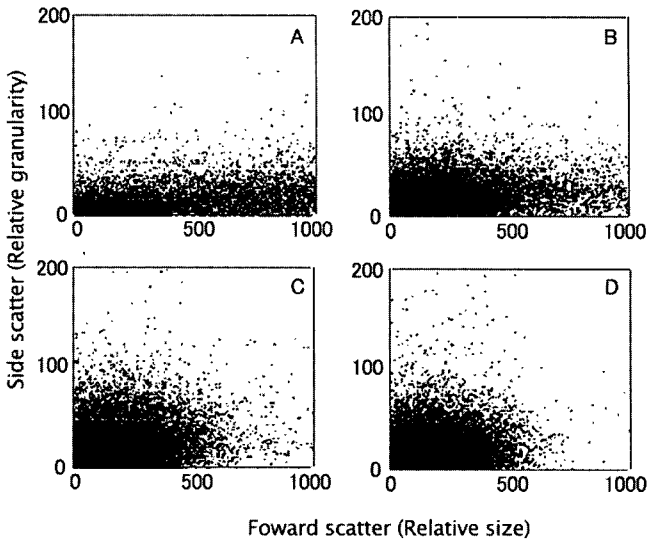

Fig. 2. Change of size of liposomes examined by flow cytometry, following bacterial reactions.
(A) *L. monocytogenes* (NCTC 7973, hemolytic strain); (B) *L. monocytogenes* (NCTC 7973, non-hemolytic strain); (C) *E. coli* (NCIB 10772); (D) control (untreated liposomes).

intensity increases. Therefore, the result from the reaction between the liposomes and hemolytic *Listeria* is consistent with the release of entrapped dye from the liposomes. Other evidence to support this observation is seen in the forward scatter results, which show that the effective size of liposomal calcein increased following the reaction with hemolytic *Listeria* (Fig. 2). In the case of side scatter results, the decrease in the relative granularity of liposomal calcein was observed, following the reaction. A small increase in the size of liposomes was also observed when non-hemolytic *Listeria* strain was involved, while the reaction with *E. coli* did not produce any structural changes in liposomes. Therefore, fluorescence intensity and light scatter studies indicate that the hemolytic action of bacteria on the liposomes produced a modified form of liposomes that was formed by the action of the hemolytic bacteria.

To investigate the actions of hemolytic factors of *L. monocytogenes* NCTC 7973 hemolytic strain on the liposomes, electron micrographic analysis was also carried out. Figure 3A shows an electron micrograph of freshly made liposomes. When the filtered liposome-hemolytic bacteria reactant was examined, however, large aggregations of unknown lipid were observed (Fig. 3B). Therefore, the electron micrographs also indicate that the hemolytic action of bacteria on the liposomes produced a modified form of liposomes that were formed by the action of the hemolytic bacteria.

When the liposomes, treated with hemolytic bacteria, were examined by flow cytometry, the most noticeable effect on the liposomes was the change in size and

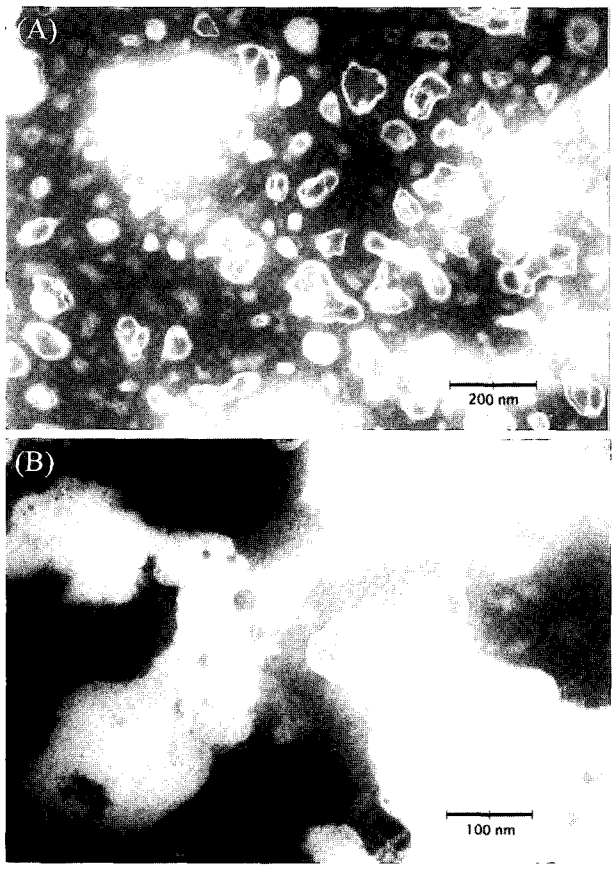

Fig. 3. Change of structure of liposomes, following bacterial reactions, examined by TEM.
(A) Untreated liposomes (bar equals 200 nm); (B) treated liposomes (bar equals 100 nm).

structure (Figs. 1 and 2). Electron microscopy analysis also indicated that the initial structure of SUVs was changed remarkably, following the treatment by hemolytic bacteria, to produce large lipid aggregations of unknown structure (Fig. 3B). In the case of *L. monocytogenes* NCTC 7973, the hemolytic strain possesses phospholipases and listeriolysin O that are associated with the pathogenicity of the organism [3]. Phospholipases appear to play an important role in releasing calcein from the liposomes, following the destruction or alteration of the liposomal structure [7]. It has been demonstrated that the catalytic activity of PLC was able to induce aggregation, and then fusion, of liposomes [1, 5, 10]. Thus, in the present study, the reason for the apparent increase in size of liposomes after reaction with hemolytic bacteria may be the aggregation and fusion of liposomes, resulting from the action of phospholipases including PLC.

Non-hemolytic *L. monocytogenes* produced a small signal in the forward scattering of flow cytometric analysis, although the signal was weaker than that obtained in the presence of hemolytic *L. monocytogenes*. The reason for this is not clear. However, in very rare occasions, non-hemolytic *Listeria* showed some pathogenicity to laboratory animals [14]. Perhaps, the organism produces small amounts of extracellular enzymes that resulted in a small signal in flow cytometer analyses. Similar action of non-hemolytic *Listeria* on liposomes was previously described [7, 16]. *Escherichia coli* (control organism) did not affect the liposomes. Further research on the action of bacterial enzymes and toxins on liposomes, by using flow cytometry, will provide rapid, simple, and valuable information relating to the virulence action of hemolytic bacteria toward mammalian cell membranes.

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