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Massive liquid crystal droplets (LCDs) have been reported in early embryogenesis and implicated in pathological progression of human diseases. The presence of LCDs has even been established as an effective diagnostic hallmark of Fabry-Anderson's disease. In this study, we report the presence of LCDs, identified by established thermal stage phase transition methods, in sputum collected during the recovery phase of respiratory infection by gram-positive bacteria. This finding provides additional insight on the breadth of liquid crystal presence in human pathology. Further study on the formation of these LCDs may lead to new perspectives on post-infection removal of infectious agents.

Keywords Gram-positive sputum; liquid-crystal like droplets; monocyte; squamous epithelial cell

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1. Introduction

Massive liquid crystal droplets (LCD) were observed in embryogenesis indicating their crucial functions in the development of embryonic liver [1,2], different developing phases of kidney [3], and yolk sac [4] and other organs [5,6]. In recent years, massive LCDs have also been discovered in many pathological processes of human diseases. In 2001, a study on lipid deposition in the human eye revealed liquid crystal and crystal like particles in Age-related Macular Degeneration (ARMD) patients, the leading cause of irreversible blindness in US [7]. Birefringent anisotropic droplets, present as Maltese crosses, were observed in the Bruch's membrane deposits, drusen, and sclera in all of 17 patients studied. Since the 1970's, liquid crystalline structures have been observed to accumulate in the special smooth foam cells of atherosclerotic lesions [7–9]. Analysis on familial hypercholesterolemia using fibroblast overloading experiments *in vitro* and on animal models have demonstrated the mechanism of lipid deposition on the vascular wall to be reliant on low density lipoprotein-cholesteryl esters complex (LDL) mediated by LDL receptors distributed on the cell surface [10–14]. This LCD accumulation of glycosphingolipids in Fabry-Anderson's disease patients caused heart disease at very high ratios compared to control subjects [15,16]. The presence of LCD has since been established as an effective diagnostic hallmark of Fabry-Anderson's disease cardiomyopathy [17].

In this study, we add to the previously documented existence of liquid crystals in disease pathology. We report that the liquid crystal like droplets were observed in sputum generated during the final stage of recovery from a respiratory infection caused by gram-positive bacteria. The LCDs, confirmed by thermal phase transition analysis, were evenly distributed in the cytoplasm of squamous epithelial cells and monocytes suspended in sputum collected during the final stage of respiratory infection. These birefringent particles were identified as LCD using previously established temperature based phase transition characteristics of liquid-crystal particles. This finding provides additional insight for exploring the breadth of liquid crystals in human pathology. Further study on the mechanism of how and when these massive cytoplasmic LCDs are initiated in the respiration system may lead to a new perspective on the biological mechanism for post-infection removal of infectious agents.

2. Materials and Methods

2.1. *Sample Preparation*

Sputum sample was collected from a 40 year old patient during the final phase of recovery from pneumonia. The sample was smeared on a slide and mounted in 20% glycerol with PBS (PH 7.4) according to standard protocol for smear preparation [1–3]. Smear-slides sealed with polymer film were preserved in $-80^{\circ}C$ for further use.

2.2. *Histology Analysis*

Hematoxylin and Eosin (H&E) staining was carried out on samples according to previously established methods [18]. After Hematoxylin and Eosin staining, slides were dehydrated in ethanol and cover-slipped with xylene based Permount medium for permanent preservation. Histology analysis was carried out under a Nikon conventional microscope (Jnoec Ltd., Jiangnan, Assembled in Nanjing, PRC).

2.3. *Gram Staining*

Fresh smear slides were allowed to air dry at room temperature before being heat fixed at 37°C for 30 minutes. Slides preserved at −80°C were allowed to come to room temperature before heat fixation. The Gram stain was used to identify bacteria in the sputum sample was performed according to the modified standard protocol [19,20]. Slides were treated with crystal violet and washed with cold water before Gram's Iodine followed by safranine was applied.

2.4. *Polarized Light Microscopy*

Conventional observations were first carried out under non-crossed polarizer and analyzer to locate and evaluate the distribution of birefringent LCDs in the sample. Optical activity of each sample was observed between two cross polarizers. Birefringence activities generated by the samples were recorded for further analysis. The observations on optical activity proceeded with a XS-213A-P Polarizing Microscope (Jnoec Ltd., Jiangnan, Nanjing, PRC) [1–4].

2.5. *Measurement of Phase Transition*

Phase Transitions were documented using both the inverted microscope PE120 peltier system (Linkam Scientific Instruments, UK) and XS-213A-P polarizing microscope [2,3]. Measurements were carried out on a 5 mm aperture PE120 peltier system at a heating/cooling rate range of 0.1 to 20°C per minute with the temperature stability regulated by RS232. Temperatures of phase transition between anisotropic and isotropic phases were recorded according to observations on birefringence activities of the samples between polarized prisms.

2.6. *Image Analysis*

Quantitative analysis of LCD birefringence intensity generating colored intensity maps was conducted with the image analysis software Image J 1.38 (NIH, Bethesda, MD).

3. Results and Discussion

3.1. *Gram Positive Identification and Histology Analysis*

The sputum contained gram-positive bacteria as characterized by the dark purple Gram stain (Fig. 1A). Histological analysis was carried out on H&E stains. This analysis revealed two major cell types present in the sputum, squamous epithelial cells (SEC) and monocytes (MC) (Fig. 1B). Squamous epithelial cells are relatively large in size with one or two nuclei, while monocytes are smaller with one nucleus.

3.2. *Identification of Liquid Crystal Droplets in Sputum*

Polarized light and differential interference contrast microscopy on samples revealed a large number of birefringent particles distributed unevenly in the samples (Fig. 1B, C, & D). The Birefringent particles are mainly located in Squamous epithelial cells and monocytes with relatively few birefringent particles present in the sputum matrix (Fig. 1E). Under 100x oil-immersion objective, the LCD can be observed as Maltese-crosses in both squamous epithelial cells and monocytes (MC) (Fig. 1

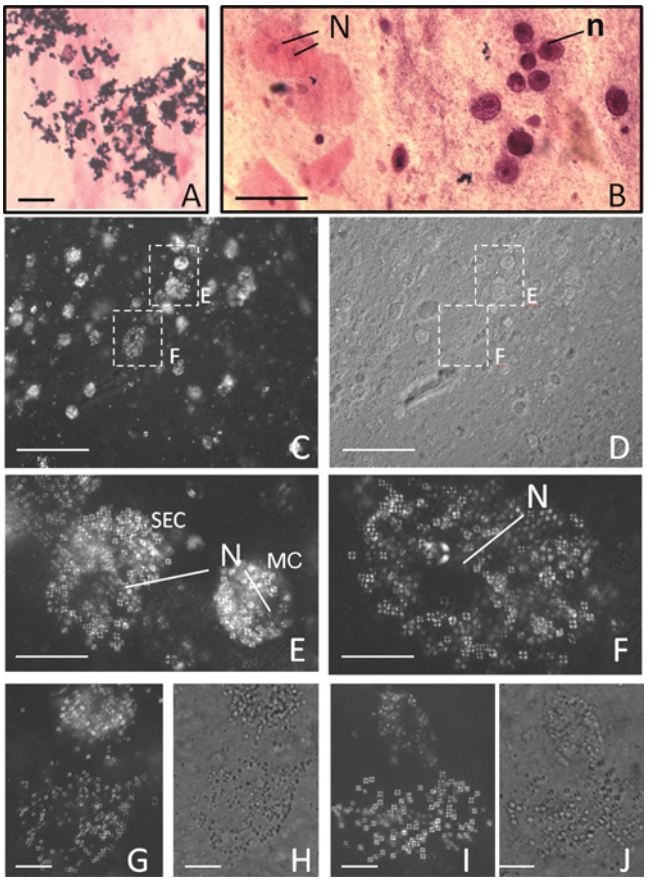

Figure 1. Histological and polarization analysis of patient sputum. Positive gram staining indicates the patient was affected by Gram positive bacteria (1A). H&E staining shows that squamous epithelial cells (SEC) make up the majority of the cells found in the sample (Fig. 1B) and monocytes (MC). Nuclei indicated with N. As observed under polarization microscope in comparison with DIC imagining (D), birefringent particles are distributed in cells found in the patient sputum (C), including SEC and MC (E and F), but not found in the sputum matrix. Birefringent Maltese-crosses are situated in the cytoplasm of both single nuclear (G and H) and double nuclei cells (top cell in Fig. 1I and J). The lengths of scale bars are 20 μm in A, B and E; 60 μm in C and D; and 10 μm in F to J. (Figure appears in color online.)

F through J). The cytoplasmic LCDs are abundantly present in both single nucleus (Fig. 1G and H) and double nuclei cells (Fig. 1I and J).

The LCDs were only observed in sputum collected during recovery from respiratory infection. Sputum samples collected during one-month and four-month follow up examinations contained no birefringent particles.

3.3. *Temperature Facilitated Phase Transitions of Cytoplasmic LCD in Sputum Sample*

The birefringent particles found in the cytoplasm of squamous epithelial cells and monocytes as well as suspended in the sputum matrix exhibited typical

temperature-based phase transition characteristic of liquid crystals. In our *in vitro* experiment, these anisotropic droplets, observed as Maltese crosses, converted to non-birefringent isotropic droplets at temperature greater than 46°C. The isotropic droplets then reverted back to Maltese cross birefringent anisotropic droplets when temperature resumed to below 46°C, suggesting that the birefringent particles observed are liquid-crystal in nature.

The transition process from Maltese cross birefringence (liquid-crystalline, anisotropic state) to isotropic droplets with temperature increase is documented in Figure 2, panel A to L. The corresponding return to birefringence with temperature decrease is recorded in Figure 2, panel M to R. The phase transition of each individual Maltese cross occured quickly and in concert with surrounding Maltese crosses found in both squamous epithelial cells. However, the LCDs found in monocytes (top-left corner of each panel) have lower phase transition temperature than those found in squamous epithelial cells. The temperature for optical transition of the LCDs in squamous epithelial cells is 46°C and 42°C for those found in monocytes.

The smear slides preserved at −80°C did not exhibit Maltese cross birefringence. Instead, crystal bundles were found in their place and stayed in the crystal in room temperature. Subsequent heating recovered Maltese cross birefringence prior to the temperature sensitive conversation into isotropic droplets at higher temperatures, demonstrating that the LCDs found in the sputum sample are capable of transitioning between crystal, Maltese crosses liquid-crystal, and isotropic states. This phenomenon is very similar to those previously reported in the embryonic liver and kidney [1,3].

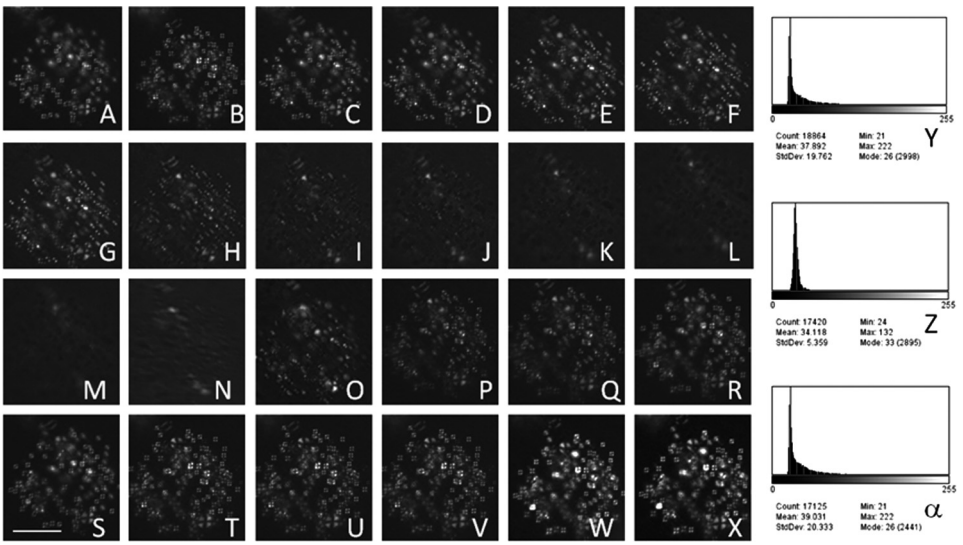

Figure 2. Phase transition of cytoplasmic liquid-crystal particles under polarized microscope. The cytoplasmic birefringence particles exhibit Maltese-crosses while incased in the cell (A). The birefringent activity of the Maltese-crosses is shown gradually decreasing during thermal-stage facilitated temperature increase (A to I) until disappearance (J to M). With temperature recovery, the birefringent Maltese-crosses eventually resumed its original birefringence (N to X). The rate of the density recovery reached 100% (Y, Z and α). The scale bar is 10 μm in length.

In Figure 2 panels S to U, representative cytoplasmic crystals display the phase transition process of crystal to isotropic droplets in sputum with temperature increase. The reverse phase transition from isotropic droplets back to Maltese cross birefringent anisotropic droplets, achieved as the droplets resumed room temperature under acceleration with a liquid nitrogen system, is documented in Figure 2 panels V to X.

3.4. *Fluidity of the Cytoplasmic LCD in Sputum*

To characterize the fluidity of cytoplasmic LCD in squamous epithelial cells and monocytes found in sputum, a pressure-recovery experiment was conducted on the smear samples. The sample was mounted in the medium described in Materials and Methods before pressure was exerted evenly on the cover-glass with a rubber pressure applicator and observations made of the LCDs between crossed polarizing prisms.

Post pressure application some Maltese Cross LCD spheres distorted into birefringent elliptical shapes (Fig. 3A to C). When the LCDs were released from the cell, birefringent elliptical (arrows Fig. 3D to E) and bowling-pin shapes LCDs (arrowheads) can be easily observed. This fluidity along with its classic liquid-crystal temperature-dependent birefringence activity, further supports our conclusion that the birefringent particles found in the cytoplasm of squamous epithelial cells and monocytes suspended in sputum generated post infection by gram-positive bacteria

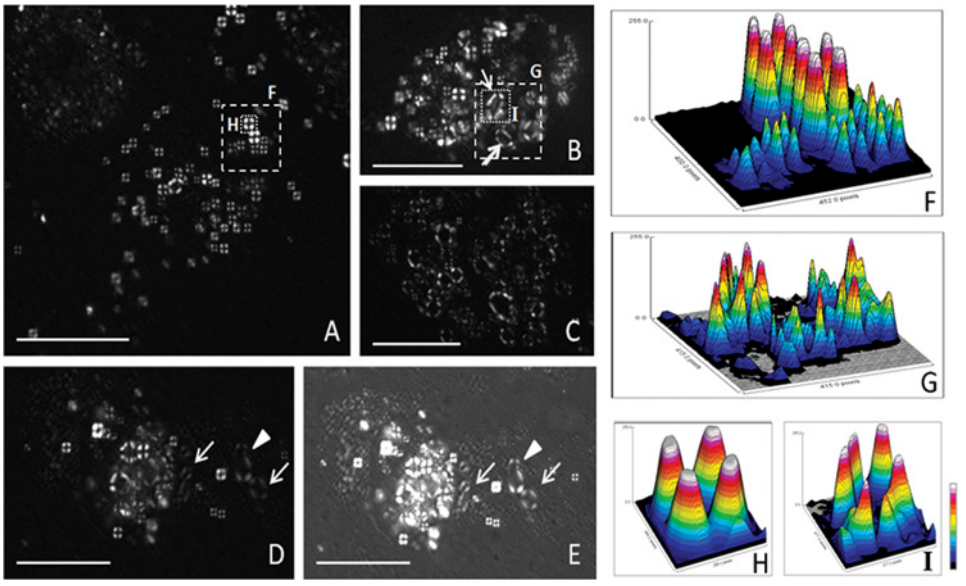

Figure 3. Fluidity of the cytoplasmic LCD in Gram positive sputum is demonstrated using a pressure-recovery experiment. Maltese-Cross LCD spheres (A) distorted into birefringent elliptical shapes under pressure (B and C). LCD released from cells ruptured during this experiment exhibited briefringent elliptical (arrows in D and E) and bowling-pin shapes LCDs (arrows-head). Panel E merges panel D with its corresponding DIC image. The density distribution of LCD before and after pressure application in panels A and B are shown in panels F and H, G and I. (Figure appears in color online.)

are liquid crystal in nature. Figure 3F and H, 3G and I show the densities of LCDs in panel A and B respectively.

In recent years, accumulation of LCDs has been found in many pathogenic tissues and organs. One such disease, Age-related Macular Degeneration (ARMD), begins with yellow deposits in macula of the eye, including the fovea and specifically in the drusen situated between the retinal pigment epithelium (RPE) and underlying choroid. These yellow LCD deposits in the eye lead to diffuse imaging. This yellow deposit was identified as a lipid stabilized in liquid-crystalline or crystal states in the deposits [9]. Mutations in HTRA1, C3 Variant, complement factor H (CFH), and ECM proteins have been linked to the disease, however it is uncertain whether these mutations lead directly to LCDs, the physical markers for the disease [21–23].

Due to limitation of the sample size, an analysis on the chemical components of the LCDs could not be performed. However, we have demonstrated through thermal-stage and pressure techniques that the birefringent particles distributed in the cytoplasm of squamous epithelial cells and monocytes suspended in sputum generated during the recovery phase of respiratory infection by gram-positive bacteria are liquid-crystalline in nature. Further study on the formation of these LCDs may lead to new perspectives on post-infection removal of infectious agents and establish new biomarkers for monitoring the progress of pneumonia caused by gram-positive bacteria.

4. Conclusions

Based on observations using polarized light microscopy, we report the presence of LCDs in sputum generated during the aftermath of respiratory infection by gram-positive bacteria. Anisotropic droplets evenly distributed in the cytoplasm of squamous epithelial cells and monocytes including macrophages suspended in sputum collected during the final stage of respiratory infection. These birefringent particles were identified as LCD using thermal phase transition and pressure distortion technique. This finding provides additional insight for exploring the reach of liquid crystals in human pathology. Further study on the mechanism of how and when these massive cytoplasmic LCDs are initiated in the respiration system may lead to a new perspective on the molecular mechanism of removing Gram positive bacteria from the body and establish biomarkers for monitoring the progress of pneumonia caused by gram-positive bacteria.

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