Original Article

EXPOSURE TO CETYL PYRIDINIUM CHLORIDE AND LOSS OF INTEGRITY OF CELL WALL OF MYCOBACTERIA

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Summary

***Background*:** Cetyl pyridinium chloride (CPC) liquefied sputum was shown to reduce AFB smear positivity presumably damaging cell wall of *M. tuberculosis.*

Settings: National Institute for Research in Tuberculosis, Chennai, (Tamil Nadu).

***Objective*:** To assess the cell wall damage of mycobacteria in CPC liquefied sputum, by Transmission Electron Microscopy (TEM) and mycobacteriophage adsorption studies.

***Methods*:** Pooled sputum sample from smear positive pulmonary TB patients was homogenized and liquefied with CPC. It was examined in TEM daily for four days, to assess cell wall damage of *M. tuberculosis*, and photomicrographs were taken. *M. smegmatis* mc^2155, treated with CPC, was infected with mycobacteriophage (phAE129) to study phage adsorption on cell wall and plaque formation. CPC untreated sputum and *M. smegmatis* formed controls.

***Results*:** Photomicrographs showed that cell wall of *M. tuberculosis* was intact in controls and damaged in CPC preserved sputum for 96 hours. Plaque formation was seen and absent respectively in CPC untreated and treated *M. smegmatis* cells.

***Conclusion*:** Exposure to CPC damaged the cell wall of *M. tuberculosis* within 96 hours. Mycobacteriophage failed to form plaques after *M. smegmatis* mc^2155 was treated with CPC implying inhibition of phage adsorption on damaged cell wall and thus providing a clue for poor staining and smear positivity in microscopy. **[*Indian J Tuberc 2013; 60:* 223-226]**

***Key words*:** *M. tuberculosis*, Cetyl pyridinium chloride, Cell wall, Mycobacteriophages, TEM

INTRODUCTION

Smithwick *et al* demonstrated the use of Cetyl Pyridinium Chloride (CPC) for the transportation of sputum samples from remote health centres to tuberculosis reference laboratories for mycobacteriological investigations[1]. WHO recommended the use of CPC for transportation of sputum samples from smear positive patients in drug resistance surveillance (DRS) to mycobacteriology laboratories.[2,3] However, liquefaction of CPC preserved sputum was found to reduce the AFB smear positivity, especially in fluorescence microscopy using auramine phenol (AP) staining.[4,5]

Scanning electron microscopy studies showed that formation of cell wall of mycobacteria was impaired due to interference of mycolic acid synthesis by INH and subsequent loss of acid-fastness of mycobacteria.[6] Phage adsorption studies provide evidence that phages infect their respective bacterial hosts by adsorbing onto specific receptors located on the cell wall of the bacteria.[7,8] When cell wall integrity is damaged due to chemical or physical pressure, phage adsorption is expected to be affected. The knowledge of possible damage of bacterial cell wall by chemicals and impairment of phage adsorption on damaged cell wall prompted us to study the cell wall damage and formation of plaques in CPC exposed *M. tuberculosis* by transmission electron microscope (TEM) and by phage adsorption studies. Reasons for reduction of AFB positivity as it is known that damaged cell wall may be limit the binding of auramine to the cell wall of mycobacteria.[9]

MATERIAL AND METHODS

TRANSMISSION ELECTRON MICROSCOPY

Pooled sputum (~25 ml) from smear positive pulmonary TB patients was homogenized in a mechanical shaker and aliquoted into five parts. The

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first aliquot was processed by modified Petroff's method[10] and formed the control. The second, third, fourth and fifth aliquots were mixed with CPC at a concentration of 7.6 mg/ml (P-CPC 38 mg for 5 ml instead of 75mg in liquid form) and kept at ambient conditions until used. Every day one aliquot was washed twice with distilled water and the deposits were each treated with 125 µl of 20% glutaraldehyde. The deposits were subjected to TEM study with negative staining by Phospho Tungstic acid (PTA) method.[11] In brief, 125 µl of 20% glutaraldehyde was added to the deposit and 2 µl of the deposit was placed on to the copper colloidion coated grid with 400 meshes. The excess was drained off after two hours. A drop of 2% PTA was added and allowed to stand for two minutes. After drying, the grid was examined under TEM (Philips Technai. 10, magnification 24000X). The morphology of bacilli was photomicrographed. The deposit from fifth aliquot was cultured onto Lowenstein Jensen (LJ) medium in addition to TEM. Suspension of *M. tuberculosis* $H_{37}RV$ (equivalent to # 1 McFarland Units) in distilled water was treated with glutaraldehyde and examined under TEM.

PHAGE ADSORPTION ASSAY

Recombinant mycobacteriophage construct, phAE129 was propagated in *M. smegmatis* mc^2155 using Luria–Bertani medium as per protocol and maintained at 4^0 C.[12] Suspension (equivalent to # 4 McFarland Units) of fresh culture (24 hours) of *M. smegmatis* (the experiment is extrapolated to *M. tuberculosis*) was made and aliquoted into two. One aliquot treated with CPC at a concentration of 7.6 mg/ml and the other treated as control were incubated at 37^0C for 24 hours. Lawns of *M smegmatis* treated with CPC and untreated were prepared by mixing 300 µl of the cells with 5 ml of soft agar (0.7%) and poured on Middle Brook 7H9 media (Difco, USA) supplemented with 5% glycerol and 10% albumin dextrose complex (G7H9) plate. Ten fold dilutions of mycobacteriophage were made up to 10^{-5} and about 5 µl from each dilution was spotted on the lawns of *M. smegmatis* mc^2155 treated with CPC and without CPC. The plates were incubated at 37^0C for 24 hours and the formation of plaques was studied.

In a separate experiment, the mycobacteriophage (titre10^9 pfu/ml) was treated with CPC (7.6 mg of CPC/ml) for two hours at 37^0C. Untreated phage was used as the control. About 5 µl of test and control phage were spotted on a lawn of *M. smegmatis* mc^2155 and incubated at 37^0C for 24 hours and the formation of plaques was studied.

RESULTS

TEM EXAMINATION OF *M. TUBERCULOSIS* IN SPUTUM TREATED WITH CPC

The cell wall of *M. tuberculosis* was intact in controls (Fig. 1a). It was increasingly distorted in sputum exposed to CPC for 24 hours, 48 hours and 72 hours (Figs. 1b, 1c and 1d). It was completely degraded after exposure to CPC for 72 hours. The

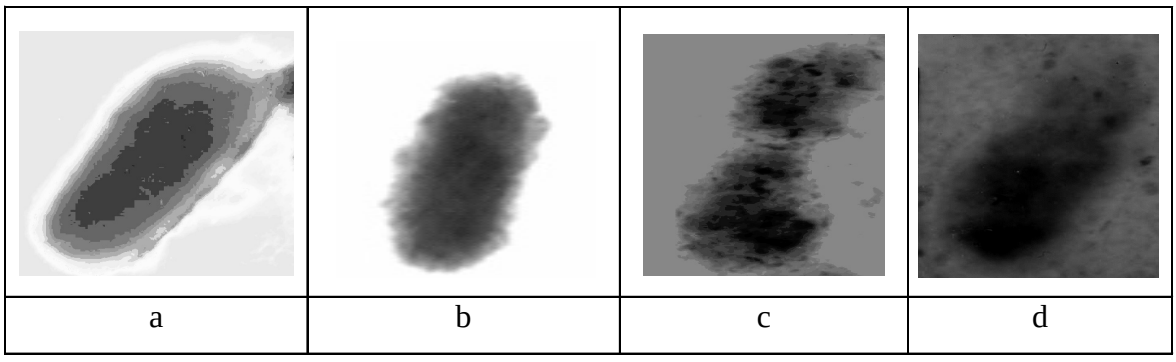

Fig. 1: Transmission Electron Microscopy photomicrographs of *M. tuberculosis* in sputum unexposed to CPC and exposed to CPC (a) Zero Day (b) 24 Hours (c) 48 Hours (d) 72 Hours

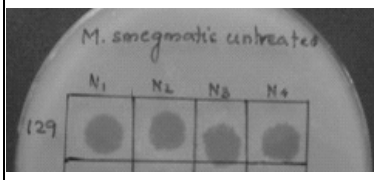	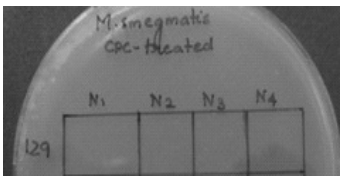	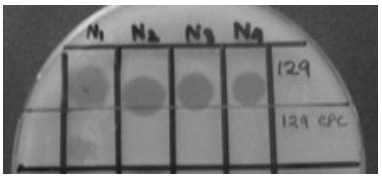
Macro plaques of phAE129 in untreated *M. smegmatis* mc^2155 lawn	No plaque formation in CPC treated *M. smegmatis* mc^2155	No plaque formation with CPC treated phAE129

Fig. 2: Phage adsorption on *M. smegmatis* mc^2155 (a) untreated with CPC (b) Treated with CPC (c) Phages treated with and without CPC

5th aliquot, washed two times with distilled water and cultured on LJ medium, grew *M. tuberculosis* (the morphology of colonies are smooth but no time difference between the growths).

PHAGE ADSORPTION ONTO *M. SMEGMATIS* MC^2155 TREATED WITH CPC

With CPC untreated cells, the lawn formation was thick and distinct macro plaques were formed (Fig. 2a). With CPC treated cells, the lawn was thin and plaques were absent (Fig. 2b). Phage exposed to CPC, did produce normal plaques (Fig. 2c).

DISCUSSION

TEM study revealed that cell wall of *M. tuberculosis* was intact both in sputum sample and in culture suspension not exposed to CPC. It also showed that the cell wall of *M. tuberculosis* was completely damaged in sputum treated with CPC for four days. Nonetheless, the 5th aliquot yielded growth of *M. tuberculosis* on LJ medium. Thus, reduction of AFB positivity in sputum preserved with CPC may be due to damage in the cell wall of *M. tuberculosis*. The damaged cell walls are deterrent to the binding of auramine. However, it is interesting to note that the bacilli with damaged cell wall still resulted in normal growth on LJ medium.

M. tuberculosis possesses unique forms of mycolic acids that are highly sensitive to isoniazid. A close relationship was shown between the synthesis of these specific mycolic acids and the staining ability of the cells.[13] Cells exposed to CPC gradually lose their integrity of cell wall until a complete loss occurred at about 72 hours.

CPC-NaCl preserved sputum yielded less AFB positives in AP method.[5] This could be due to exposure of *M. tuberculosis* to CPC. The reliability of AP method is due to more intensive binding of mycolic acids of the bacilli to auramine and that the bacilli stand out sharply against dark background to allow rapid and accurate screening under low power objective. It can be deduced from the present experiment that bacterial cell wall is damaged after exposure to CPC resulting in the poor staining ability by AP method leading to reduced smear AFB positivity.

Lindberg *et al* demonstrated that phage adsorption was affected due to impaired cell wall synthesis using cell wall deficient forms of Salmonella species.[14] David *et al* showed that addition of colistin inhibited the lytic cycle of the mycobacteriophage D29 in *M. tuberculosis*.[15] In the current study, normal plaque formation was seen in untreated cells demonstrating effective phage adsorption. There was no plaque formation in CPC treated cells. Absence of plaque formation could be attributed to the cell

wall damage in the host due to CPC exposure or inactivation of phages. The latter was tested in the second experiment in which phages were exposed to CPC and made to infect untreated normal host cells.

CONCLUSION

Exposure to CPC results in the damage of *M. tuberculosis* cell wall as observed under transmission electron microscope. Exposure of *M. smegmatis* to CPC impairs phage adsorption. The cell wall damage observed in CPC preserved sputum could be attributable to the poor staining and reduced AFB positivity in microscopy.

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