Modified Method for Evaluation of Plasma Membrane Integrity in Eukaryotic Cell

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We propose a method for evaluation of the number of viable cells by the content of bromcresol purple dye absorbed by dead cells from the incubation medium. Myramistin was used as a pore-forming agent. The number of live cells in yeast suspension inversely correlated with the percentage of dye absorbed by cells. The method is simple and requires no special equipment. The effect of myramistin on *Candida albicans* and *Malassezia sympodialis* cells and on epitheliocytes was evaluated. Two-hour treatment with myramistin in a concentration commonly used in clinical practice (100 μg/ml) decreased the number of viable cells by 2 and 1 order of magnitude, respectively. Epitheliocytes under the same conditions absorbed approximately the same mount of the dye as *Candida* cells.

Key Words: *plasma membrane; integrity; viability; yeast; epitheliocytes; eukaryotes; myramistin*

Studies of eukaryotic cells often require evaluation of their viability. The integrity of plasma membrane (PM) is an important marker of cell viability. Methods for evaluation of PM integrity are based on cell staining with dyes absorbed only by damaged cells. For instance, PM integrity in spermatozoa used for extracorporal fertilization is evaluated by propidium iodide and/or carboxyfluorescein diacetate staining [4,7]. Bromcresol purple (BCP) [6] or rhodamine [8] are used for determination of the number of dead yeast cells after exposure to yeast toxins. In any case, quantitative assessment of PM integrity requires special equipment (fluorescent microscope, flow cytofluorometer).

The method proposed by us is also based on the use of BCP dye. This method is simple and rapid, and requires no sophisticated equipment.

MATERIALS AND METHODS

Yeast cells and epitheliocytes were taken as the objects of investigation. Pure *Candida albicans* culture was obtained by inoculation of bronchial secretion from an asthmatic patient on a glucose-peptone-yeast medium with antibiotic [1]; the species was identified according to a classical protocol [9]. *Malassezia sympodialis* yeast was isolated from normal human skin by inoculation into modified Dixon medium; the species was determined as described previously [5]. Epithelial cells were obtained by scraping normal human oral mucosa.

The antiseptic myramistin (benzyldimithin[3-(myristoylamino)propyl]ammonium chloride, a surface-active agent loosening cell membrane [2,3]) was used as the pore-forming agent.

Absorption of stained cell suspensions and dye solutions (BCP) was measured at pH 4.6 and λ=440 nm, which corresponded to its maximum absorption at this pH [6].

Different approaches to quantitative evaluation of PM integrity using stains penetrating through ionic channels are practiced. The traditional approach consists in counting of stained/unstained cells. Another approach consists in measuring of the amount of dye by absorption of cell suspension (compared to control unstained suspension) or by the amount (concentration) of dye entering the cells.

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We compared these three approaches using myramistin in different concentrations as the pore-forming agent. The first step in these studies was cell treatment with myramistin.

Yeast cells in the stationary growth phase (optical density was adjusted to 3 at λ=540 nm) were treated with myramistin for 2 h at 27-30°C on a shaker. The concentration of myramistin varied from 0 (control) to 1000 μg/ml. Then the cells were washed twice with 0.5 M phosphate buffer (pH 4.6) and precipitated by centrifugation at 3000*g*. The precipitate was suspended in 4 ml of the same buffer with 100 μl 10 mM BCP and incubated for 1 h under constant stirring, after that it was precipitated again and optical density of the supernatant was measured. Cell precipitate after staining was colored from creamy (control) to dark brown (at a concentration of 1000 μg/ml).

RESULTS

Microscopic examination of the precipitate showed that the color of cells in the studied dye concentration range varied from white to yellow-brown. However, these variations reflected the intensity of cell staining, but not the number of stained cells. Hence, this approach cannot be effective in all cases.

The staining intensity in cell suspensions varied from 35 to 57% because of the presence of cell agglomerations and high rate of cell precipitation during measurements of optical density.

Evaluation of the amount of dye in cells by the difference between the initial and final content in the medium was the most accurate and productive method. This method requires no additional washout after staining. The degree of PM loosening can be expressed as the percent of the total amount of dye absorbed by cells and calculated by the formula:

$$\frac{OD_C\text{-}OD_T}{OD_C} \times 100\%,$$

where ODC and ODT are optical densities of the supernatant without treatment and after treatment with myramistin, respectively.

The higher is the percentage of absorbed BCP, the higher is pore-forming activity of the test agent towards this cell suspension.

This method was used for evaluation of the pore-forming effect of different myramistin concentrations on stationary *Candida albicans* and *Malassezia spp.* cultures and on epitheliocytes. The dye absorption through PM pores in cell suspensions with initially similar density differed significantly. Saturation of *Candida albicans* cells was attained at a myramistin concentration of 500 μg/ml. Further increase in its concentration did not increased dye absorption. For *Malassezia spp.* even the concentration of 1000 μg/ml was not saturating (Fig. 1).

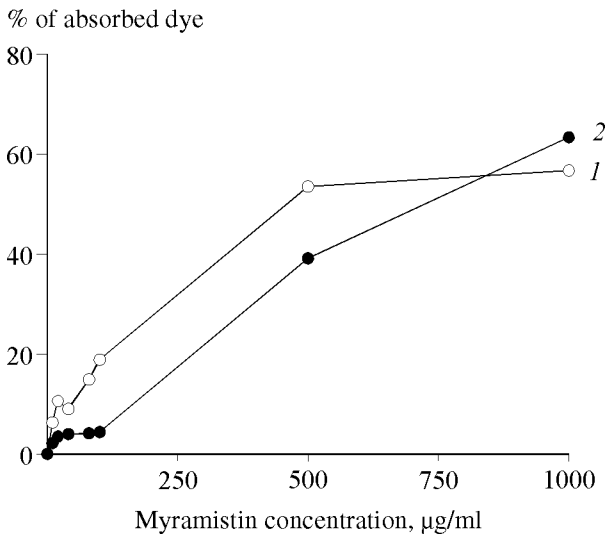

Fig. 1. Pore-forming effect of myramistin on *Candida albicans* (*1*) and *Malassezia spp.* (*2*) evaluated by absorption of bromcresol purple.

At myramistin concentration of 100 μg/ml the number of viable *Candida* cells decreased by 2 orders of magnitude compared to the control, while the number of viable *Malassezia* cells decreased only by one order of magnitude under the same conditions (Table 1). Myramistin in a concentration of 1000 μg/ml killed all *Candida* cells and reduced the number of viable *Malassezia* by only 2 orders of magnitude, which was confirmed by measurements of BCP staining intensity (Fig. 1). Pierson correlation coefficient reflecting the linear relationship between the percentage of dye absorbed by cells and number of viable cells was -0.763 for *Candida* and -0.650 for *Malassezia*, *i.e.* the more dye was absorbed by cells, the lower was the content of viable cells in the suspension.

We compared the effect of myramistin on yeast cells with its effect on epithelial cells isolated from normal human buccal mucosa. Optical density of epitheliocyte suspension was adjusted to that of yeast suspension. The effect of the test agent in a concentration used in clinical practice (100 μg/ml) was studied. It was found that the concentration of BCP absorbed by epitheliocytes was 18±3%, while for *Candida albicans* and *Malassezia spp.* this parameter was 17±2 and 4±1%, respectively. We concluded that long-term treatment of the mucosa with myramistin can induce epitheliocyte death.

Hence, the proposed modified method allows rapid and accurate evaluation of PM integrity, the parameter reflecting viability of eukaryotic cells. The method requires no special equipment and is easy to use.

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