Discrimination of live, anti-tuberculosis agent-injured, and dead Mycobacterium tuberculosis using flow cytometry

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Abstract

Flow cytometry (FCM) using propidium iodide (PI)/bis-oxonol (BOX) staining can distinguish live, dead, and sublethally injured Escherichia coli by detecting intact vs. nonintact membranes (PI) and membrane potential (BOX). However, live bacteria, especially Mycobacterium tuberculosis, are not likely to be successfully discriminated from injured bacterium by FCM when utilizing the live/dead staining agents currently on the market. As injured cell membranes have integrity like that of live cells and are regarded as such by FCM, the distinction between live and injured cells has depended on the culture method, where injured bacteria cannot grow in general. We have previously shown that photoactivated ethidium monoazide (EMA) directly cleaves bacterial DNA both in vivo and in vitro. In this study, we found that the chromosomal DNA of antibiotic-injured, but not live, M. tuberculosis could be cleaved within 2 h by EMA, and that the resultant decrease in the spaces of DNA base pairs could greatly inhibit the intercalation of SYTO9 in FCM. The percentage value of SYTO9+/PI−/C0− quadrant from antibiotic-injured M. tuberculosis after EMA treatment decreased by at least 80%, compared with that before EMA, but such a phenomenon did not take place in live cells. FCM (SYTO9/PI) following EMA treatment is a very rapid, simple, and effective method for discriminating live, antibiotic-injured, and dead M. tuberculosis without culture.

Introduction

Detecting injured cells has been a highlighted issue in nutritional, clinical, and food hygienic areas (Gunasekera et al., 2000; Amor et al., 2002). The distinction of live, sublethally injured, and dead Escherichia coli by flow cytometric (FCM) analysis has been limited to the use of propidium iodide (PI)/bis-oxonol (BOX) (Hewitt & Nebe-Von-Caron, 2001). FCM using commercial live/dead staining reagents other than PI/BOX cannot distinguish live bacteria and bacteria injured by a variety of stresses such as low pH or temperature (Bunthof & Abee, 2002). This is because the cell walls of injured bacteria remain physically intact. When the culture method is simultaneously utilized for discerning live from injured bacteria with FCM (Bunthof & Abee, 2002), this becomes time consuming. Furthermore, there have been no reports discriminating live, injured, and dead Mycobacterium tuberculosis using FCM. Live M. tuberculosis in tuberculosis patients becomes injured or dies if anti-tuberculosis agents (ATA) are administered at a certain point. Rapid assignment of live and injured M. tuberculosis by FCM is very significant because currently it takes c. 1 month to judge live or injured M. tuberculosis using culture after FCM analysis.

Ethidium monoazide (EMA) (Hixon et al., 1975; Bolton & Kears, 1978; Yielding & Firth, 1980; Graves et al., 1981) is utilized in a live/dead staining (Riedy et al., 1991) for eukaryotic cells. For prokaryotic cells, EMA binds irreversibly to the chromosomal DNA of dead, but not live, bacteria by covalent attachment in a short time (Nogva et al., 2003; Rudi et al., 2005), although ethidium bromide reversibly intercalates DNA (Tomchick & Mandel, 1964; Waring, 1965). We reported on the function of EMA directly cleaving chromosomal DNA in heat-injured bacteria without mediating enzymes retained in bacteria (Soejima et al., 2007) and have developed a technique detecting live bacteria alone during PCR amplification (Soejima et al., 2008). In this study, we demonstrate that DNA cleavage could take place in
ATA-injured bacteria following treatment with EMA and successive irradiation by visible light (EMA+light), and that the direct DNA breakages extensively suppress SYTO9 intercalation.

Materials and methods

Reagents

EMA was purchased from Sigma (St. Louis, MO). SYTO9/PI staining agent (LIVE/DEAD BacLight™ Bacterial Viability Kit; Molecular Probes, Eugene, OR) was utilized in live/dead staining. Isoniazid, rifampicin, streptomycin, and kanamycin (Sigma) were used to prepare the ATA-injured cells of M. tuberculosis.

Bacteria and culture

Mycobacterium tuberculosis H37Ra cultivated in Ogawa medium slant (Kyokuto Seiyaku, Tokyo, Japan) was subcultured in Sauton broth containing 0.05% Tween 80 (Sauton-Tw80) at 37 °C for a week. An aliquot of 10 mL of the logarithmic growth phase was centrifuged at 8000 g for 15 min at 4 °C. Saline 10 mL containing 0.05% Tween 80 (saline-Tw80) was added to the bacterial pellets to prepare the live suspension of M. tuberculosis. Middlebrook 7H10-agar (Becton Dickinson, Franklin Lakes, NJ) was utilized for measuring OD600 nm and for enumerating CFU.

Preparation of ATA-injured and dead M. tuberculosis

Full-growth culture (2 mL) of M. tuberculosis in Sauton-Tw80 was subcultured to 200 mL of fresh Sauton-Tw80, and then rifampicin (150 mg mL⁻¹, 200 μL) plus isoniazid (10 mg mL⁻¹, 100 μL), rifampicin (150 mg mL⁻¹, 200 μL) plus streptomycin (100 mg mL⁻¹, 400 μL), or streptomycin (100 mg mL⁻¹, 400 μL) plus kanamycin (100 mg mL⁻¹, 400 μL) were added. The suspensions were incubated at 37 °C for 3 months to either injure or completely kill the live cells of M. tuberculosis, and were centrifuged at 8000 g for 15 min at 4 °C. After washing, they were suspended in 20 mL of saline-Tw80. SYTO9 but not PI, penetrated into the ATA (rifampicin+isoniazid, rifampicin+streptomycin, and streptomycin+kanamycin)-treated cells but these cells could not form colonies and were defined as ATA-injured M. tuberculosis, since Bunthof & Abe (2002) defined ‘cells intact and metabolically active but not culturable due to various stress’ as ‘injured cells’ by the FCM and culture methods. The injured suspensions were counted by plotting a standard curve with OD600 nm values vs. live counts.

To prepare heat-killed bacterial suspensions, live bacterial suspensions were soaked in a boiling water bath for 32 min. No colony was detected by culture, but SYTO9 and PI permeated the cells as shown by FCM, which fulfilled the definition of dead M. tuberculosis (Bunthof & Abe, 2002).

Assays for ATP content and esterase activity in M. tuberculosis

ATP in the bacteria was measured using a Lucifer ATP extinction reagent set and Lucifer 250 plus (Kikkoman, Noda, Japan) following the manufacturer’s instructions. The esterase activity of the bacteria was measured by FCM using carboxyfluorescein diacetate (cFDA) (Molecular Probes Europe BV, Leiden, the Netherlands) and PI (Amor et al., 2002).

EMA treatment and irradiation with visible light

Preparation of EMA solution and successive visible light irradiation were performed according to our previous reports (Soejima et al., 2007, 2008). To distinguish ATA-injured from live M. tuberculosis, each M. tuberculosis suspension (1 mL) was treated with EMA (10 μg mL⁻¹ of final concentration; 4 °C for 2 h in the dark) and visible light irradiation (10 min).

In vitro evaluation of the EMA effect on the SYTO9 intercalation of DNA

Chromosomal DNA of M. tuberculosis purified with ISOPLANT (Nippon Gene Co. Ltd, Tokyo, Japan), 100-bp DNA ladder, and λ-EcoT14 I digest (Takara-Bio, Ohtsu, Japan), as controls, were gel-electrophoresed in 0.8% agarose gel. Then the following procedures were carried out: (1) the gel was soaked in 100 mL of 10 μg mL⁻¹ EMA solution at 4 °C for 30 min in the dark; (2) visible light irradiation for 10 min; (3) washing with sterile water; and (4) the gel was immersed in 20 mL of 0.5% TAE buffer supplemented with 30 μL of 3.34 mM SYTO9 for 30 min under the safelight. An experiment was carried out in which the order of EMA and SYTO9 treatment was reversed. After the procedures were carried out, the gel was set on the UV transilluminator (λ260 nm; densitograph (ATTA Corp., Tokyo, Japan) and a photograph of the gel image taken using a digital camera.

Fluorescent microscopy

SYTO9/PI solution (3 μL, ratio 1:1) was added to 1 mL of the M. tuberculosis suspension (1 mL) and kept at room temperature for 15 min in the dark. Fluorescence microscopy (60 objective lens) was performed with confocal scanning system radiance 2100 AGR 3 (Biorad, Hemel Hempstead, UK).
A 1-mL *M. tuberculosis* suspension, before and after EMA light treatment, was centrifuged at 8000 g for 15 min at 4 °C, the supernatant was removed, and then 1 mL of saline-Tw80 was added to the pellet. SYTO 9/PI 3 mL was added, then FCM measurement was done, utilizing an FACS Calibur flow cytometer (Becton Dickinson, Sydney, Australia). An argon laser at 15 mW (λ488 nm) was utilized as excitation. Forward scatter (FSC, < 15°), side scatter (SSC, > 15°) and three fluorescence signals (FL1, 530 nm; FL2, 585 nm; FL3, 670 nm) were measured. The threshold was 150 (FSC signals) and the sending rate 12 μL min⁻¹. The upper limit for cells collected in the bacterial gate (FSC: 10^2 to 2 × 10^5; SSC: 10 to 2 × 10^3) was 5000 000, but the measurements were normally stopped within 30 s, with cells of 7668–13 522 harvested. The detector set was: FSC, E02; SSC, 376; FL 1, 709; FL 2, 736; FL 3, 811 using logarithmic gains. The percent compensation was shown as FL 1–FL 2 = 0.0; FL 2–FL 1 = 0.0; FL 2–FL 3 = 0.0; FL 3–FL 2 = 0.0.

### Results

#### Characterization of ATA-injured *M. tuberculosis* by ATP bioluminescence or esterase assay

Table 1 shows the ATP content in live, ATA-injured, and dead *M. tuberculosis* classified using FCM (SYTO 9/PI) and culture (Bunthof & Abee, 2002). The ATP contents of three kinds of ATA-injured cells were significantly lower than those of live cells (*P* < 0.05) but were significantly higher than those of dead cells (*P* < 0.05) using the one-way layout ANOVA, and Bonferroni, Tukey and Scheffe tests.

Esterases of live, ATA-injured, and dead *M. tuberculosis* are shown in Fig. 1. The esterase activities (cFDA) of the three pairs of ATA-injured cells were almost the same as shown as FL 1–FL 2 = 0.0; FL 2–FL 1 = 0.0; FL 2–FL 3 = 0.0; FL 3–FL 2 = 0.0.

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**Table 1. ATP assay of live, antibiotic-injured, and dead *Mycobacterium tuberculosis***

<table>
<thead>
<tr>
<th>Antibiotic-injured</th>
<th>Dead</th>
<th>NC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3100</td>
<td>180</td>
<td>1900</td>
</tr>
</tbody>
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Results are mean ± SD of three independent experiments.

*NC indicates negative control without any organism, that is, only saline-Tw80.

*1Turbidity OD₆₀₀nm was adjusted to same among the group.

*2ATP content was presented as relative light unit (RLU).

INH, isoniazid; RIF, rifampicin; STR, streptomycin; KAN, kanamycin.

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**Fig. 1.** Esterase assay of live, antibiotics-injured, and dead *Mycobacterium tuberculosis*. The x-axis shows the intensity of green fluorescence (FL1) stemming from degradation of substrate cFDA by esterase. The y-axis indicates the intensity of the red fluorescence being released from PI by argon laser (λ488 nm). Live suspension counts were performed using 7H10-plate agar. Injured suspensions were enumerated using a standard curve with OD₆₀₀nm values vs. live counts. Live cells were soaked in a boiling water bath to prepare dead cells. Cell concentrations were all adjusted to c. 5.2 × 10⁶ cells mL⁻¹. The measurements were performed in duplicate, and plots were similar; thus representative data was disclosed. Numbers in quadrant are percent of plots.
those of dead cells, but were obviously lower than those of live cells.

**Influence of EMA on the SYTO9 intercalation of DNA in vitro**

Figure 2 displays gel-electrophoresed DNA bands after excitation with the UV transilluminator ($\lambda_{260\,nm}$). The DNA bands stained with SYTO9 emitted green fluorescence originating from SYTO9 (Fig. 2a). Next, DNA cross-linked and cleaved by EMA and light was stained with SYTO9. The emission of red fluorescence derived from EMA was detected (Fig. 2b). The fluorescent color of the bands was the same as DNA without SYTO9 staining (Fig. 2c), which means SYTO9 did not intercalate DNA after treatment with EMA+light. When DNA intercalated by EMA was stained with SYTO9, DNA bands showed a faint whitish color as a result of the mixture of green and red (Fig. 2d). SYTO9 staining after washing EMA showed the same phenomenon as staining without washing (data not shown). Furthermore, when DNA that was first intercalated by SYTO9 was treated with successive EMA+ and light−, parts of the bands emitted whitish fluorescence; partly weak red fluorescence stemming from EMA was also emitted from the bands (Fig. 2e).

**Fluorescent microscopy of live and ATA-injured M. tuberculosis combined with EMA+light**

Figure 3 shows fluorescent microphotographs of *M. tuberculosis* stained by SYTO9 and PI before (Fig. 3a–e) and after (Fig. 3f–j) EMA+light treatment. Live and ATA-injured cells of *M. tuberculosis* could not be discriminated without EMA+light treatment (Fig. 3a–e). After EMA+light (Fig. 3f–j), the green fluorescence from live cells remained, but the degree of greenness of the three pairs of ATA-injured *M. tuberculosis* decreased greatly. The intensity of red fluorescence from dead *M. tuberculosis* also decreased.

**Distinction between live and ATA-injured M. tuberculosis using FCM combined with EMA+light**

The upper samples supplied for fluorescent microscopy were analyzed using FCM, and the results are displayed in Fig. 4a–j. Live *M. tuberculosis* and *M. tuberculosis* ATA-injured by SYTO9/PI staining before treatment with EMA+light were plotted in an area of SYTO9+ and PI−, but dead *M. tuberculosis* were set in SYTO9− and PI+. The discrimination between dead and live/ATA-injured *M. tuberculosis* was successfully performed, but that between live and ATA-injured *M. tuberculosis* was not distinct.
After performing EMA + light, the SYTO9 intensities of ATA-injured *M. tuberculosis* decreased to between 1/10 and 1/100, compared with the organism before EMA + light (Fig. 4b–d, g–i). Thus, the simultaneous distinction between live, ATA-injured, and dead *M. tuberculosis* was clearly made only with FCM (SYTO9/PI) combined with EMA + light. These results demonstrated that the injured *M. tuberculosis* could apparently be discriminated...
from live cells by FCM (SYTO9/PI) plus EMA+light, without culturing the organism.

**Discussion**

There have been no reports that demonstrate a successful distinction between live and ATA-injured *M. tuberculosis* using the FCM method. The situation is the same in other genera (Bunthof & Abee, 2002).

As shown in Fig. 4 (SYTO9+/PI− quadrant), EMA+light decreased the plots (%) of ATA-injured *M. tuberculosis* to <17.3%, compared with the percentage of the same quadrant before EMA+light (>81.5%). On the other hand, quadrant (%) of SYTO9+/PI− of three ATA-injured *M. tuberculosis* increased sixfold after EMA+light, at least compared with before EMA+light. Hence, the discrimination

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**Fig. 6.** Scheme for discriminating live, ATA-injured, and dead *Mycobacterium tuberculosis* using FCM (SYTO9/PI) before and after treatment with EMA+light. ATA: anti-tuberculosis agent-injured.
between live and injured cells was achieved within 3 h. Although the plots of live, ATA-injured and dead \textit{M. tuberculosis} partially overlapped, we were able to discriminate among three physiological states after EMA+light (Figs 4f–j and 5).

When ATA are effective against \textit{M. tuberculosis}, the bacteria is injured or killed. Culture methods, which take more than 1 month to see the results, only show the presence of live and culturable bacteria, but cannot judge the effectiveness of ATA. Our method, FCM (SYTO9/PI) plus EMA+light, could clearly distinguish between live and ATA-injured \textit{M. tuberculosis} within 3 h (Fig. 6).

The quadrant (%) of SYTO9+/PI− for ATA-injured \textit{M. tuberculosis} by EMA+light decreased by at least 80% (Fig. 4b–d, g–i). It is conceivable that SYTO9 could not intercalate DNA cross-linked and cleaved by EMA (Fig. 2a–c). As the intact spaces formed between base pairs of DNA were greatly decreased by the direct cleavage and cross-linking function of EMA (Soejima \textit{et al}, 2007), SYTO9, as a post staining agent, could not intercalate the spaces of DNA as shown in \textit{in vitro} examination (Fig. 2).

Differences in the permeability of EMA to cell membranes must be highlighted to discriminate live, ATA-injured and dead \textit{M. tuberculosis} using FCM. The principle of live/dead staining agents in FCM is based on the difference in membrane penetration by SYTO9 (green color) and PI (red color). SYTO9 permeates both live and dead bacteria due to its high permeability, but PI can only penetrate dead cells where membrane damage is severe (Fig. 6). In the case of \textit{M. tuberculosis}, SYTO9 penetrated all states of the bacteria, but PI only entered dead \textit{M. tuberculosis}. On the other hand, although EMA could not permeate live \textit{M. tuberculosis}, it could penetrate ATA-injured \textit{M. tuberculosis} including dead cells (Fig. 4), which also indicates that the degree of membrane permeability of EMA is between that of SYTO9 and PI (Fig. 6).

EMA has been used as both a cross-linking agent and a staining agent for dead eukaryotic cells, that is, the classical usage is close to that of PI, which stains dead bacteria. It is conceivable that EMA binds to DNA of dead cells by irreversible covalent attachment, but PI simply intercalates DNA of dead cells by reversible attachment. We previously reported a direct DNA-cleaving function by EMA+light (Soejima \textit{et al}, 2007) and a method to detect only live bacteria during PCR amplification (Soejima \textit{et al}, 2008). In the present study, we could rapidly discriminate, by FCM analysis, live and ATA-injured \textit{M. tuberculosis} using EMA+light through cross-linking and breaking the DNA of injured \textit{M. tuberculosis}. Because EMA is irradiated with strong visible light through the direct DNA-cleaving procedure, EMA does not work as a DNA staining agent when the excitation wavelength was set at 488 nm. This is the great difference between our use and the classical use of EMA.

Lastly, we compared the discriminatory ability of the ATP/esterase method and ours. The ATP method can precisely discriminate live, ATA-injured, and dead \textit{M. tuberculosis} of FCM+culture, but the method cannot quantify the populations of the three states. For the esterase assay, ATA-injured \textit{M. tuberculosis} could not be discriminated from dead cells and, as a result, could not precisely measure the three populations, even if PI was simultaneously used (Fig. 1). The discriminatory ability of our method is conceivably parallel to that using FCM (SYTO9/PI) combined with culture (Bunthof & Abee, 2002). Our method is more precise in discriminating live, injured, and dead \textit{M. tuberculosis} than the ATP and esterase assay method.

Acknowledgement

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References


Riedy MC, Muirhead KA, Jensen CF & Stewart CC (1991) Use of a photolabeling technique to identify nonviable cells in fixed homologous or heterologous cell populations. \textit{Cytometry} 12: 133–139.


