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Use of the Fluorescent Staining Method for Determining the Viability of *Mycobacterium lepraemurium*

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ABSTRACT

The cell suspension of *Mycobacterium lepraemurium* was exposed to heating of 40°C to 70°C for various lengths of time. The percent green-fluorescent cell by the midified fluorescein diacetate (FDA) -ethidium bromide (EB) staining method was calculated and compared with the infectivity to mice. The reduction in the percentage was associated significantly with the loss of the infectivity.

The feasibility of using the fluorescein diacetate (FDA)-ethidium bromide (EB) staining method for evaluating the viability of mycobacterial cells has been reported[3-5]. Theoretically, intact cells appear stainded green under ultraviolet illumination and are distinguished from dead and damaged cells which stain red-orange[2,6]. Kvach and Veras[5] reported that the results of their FDA/EB staining method with cultivable mycobacteria were paralleled to those of the viable units by cultivation, but they could not clearly differentiate *Mycobacterium leprae* cells by the method mainly because of the momentary fading of green fluorescence under fluorescent microscopy. We improved their method[7], and consequently the cells of either *M. leprae* or *Mycobacterium lepraemurium* were stained clearly. However, no information is available as to whether the green-stained *M.lepraemurium* cells are viable while the others are dead. The purpose of this research was to apply our improved FDA/EB method to enumerating the viability of *M. lepraemurium* cells. The cells of *in vivo* grown *M. lepraemurium* were obtained from the liver of a mouse 6 months previously infected intravenously with the Hawaiian strain of the organism. A single cell suspension was prepared with Dubos Tween-albumin-liquid medium (DLM) (Eiken Kagaku Co., Tokyo, Japan) by the method previously described[7]. The cell suspension was exposed to temperatures indicated in Fig. 1 for various lengths of time, and stained

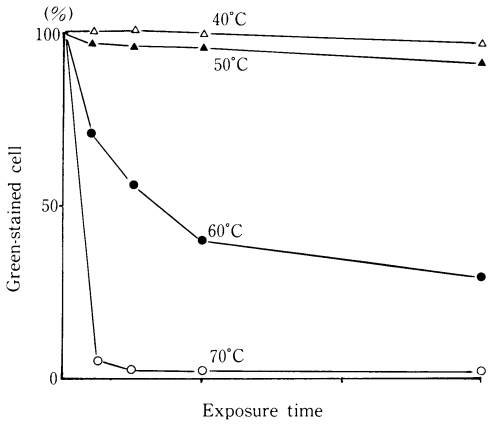

Fig. 1. Effect of heating on the percent green stained cell of *M. lepraemurium* as determined by the modified FDA/EB method. The percent was expressed as percentage of the value of starting material.

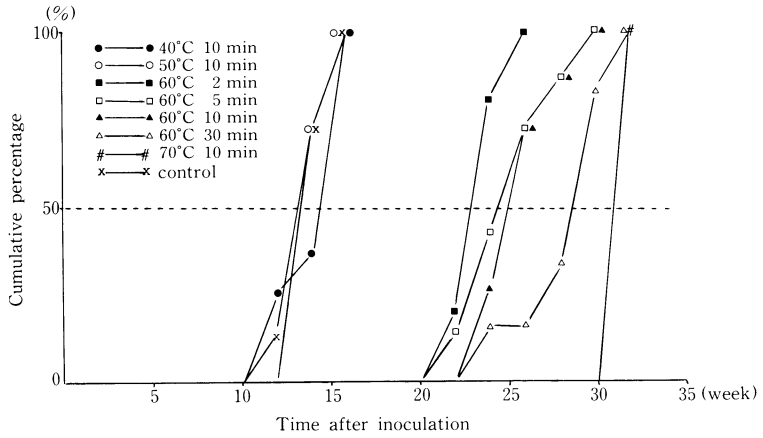

Fig. 2. Cumulative percentage of leproma formation in CBA/JNCrj mice after inoculating heat-treated *M. lepraemurium*.

by the FDA/EB working solution in which per ml 100 μg FDA and 8 μg EB were incorporated. The percentage of green stained cells was calculated and was expressed as the relative percentage of the starting material. One million cells of each suspension thus treated were inoculated subcutaneously at the breast of CBA/JNCrj mice (Japan Charles River Co. Ltd., Atsugi, Japan). The developement of murine leproma at the site of inoculation was examined every one or two weeks. As shown in Fig. 1, a reduction in percentage of green-stained cells was not significant during the heating between 40°C and 50°C up to 30 min, while it was proportional to the amount of time of exposure to 60°C and 70°C. Fig. 2 shows the course of development of leproma after inoculation with heat-treated *M. lepraemurium* cell suspensions. The leproma formation delayed according to degrees of temperature exposed as well as the amount of exposure time, indicating a decrease in infectivity of the organism. The relationship between the results of FDA/EB staining and those of infectivity was analyzed: in Fig. 3, an adequate correlation was demonstrated ($p<0.01$).

The major points of our improved FDA/EB method compared with the original one by Kvach and Veras[5] are as follows. The first point is the procedure for preparation of cell suspension. For washing and suspending the bacterial cells, DLM is used instead of Hanks' balanced salt solution. According to Hanks[1], cells of intracellular microorganisms including *M. lepraemurium* retain only vestiges of their normal metabolic activity by the time they have been washed free from the host components, but this deterioration is not due directly to loss of viability. DLM, especially purified albumin fraction included in it, might have prevented the leaching of *M. lepraemurium* cells during washing or dilution and allowed the viable cells to keep the accumulated green fluorescence within the cells. The second point is the concentrations of FDA and EB in the working staining solution. The final concentrations of FDA and EB were 50 times and 2 times as high as the original formulation, respectively. The third point is the staining. The sample mixed with the working solution was incubated for 50 to 60 min at 37°C in place of 10 to 45 min at room temperature.

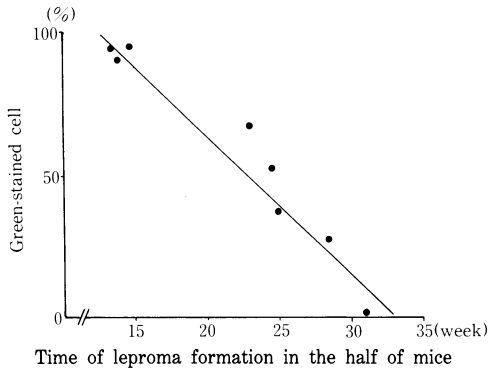

Fig. 3. The relationship between the results of FDA/EB staining and those of infectivity of *M. lepraemurium*.

Our ultimate goal of this research is to determine whether the FDA/EB staining can be used to accurately monitor the viability of *M. leprae.* As far as the present result is concerned, our improved method permits the cells of *M. lepraemurium* to be stained qualitatively equal to cultivable mycobacteria and a reduction in percent of green-stained cells is consistent with the loss of infectivity. The FDA/EB staining method would be an alternative one for evaluating non- or hard cultivable mycobacterial cells.

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