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Susceptibilities of *Mycobacterium leprae* and *M. avium* Complex to the H_2O_2-Fe-mediated Halogenation System Supplemented with Antimicrobial Agents[1]

Haruaki Tomioka, Yoshitaka Yamada, Hajime Saito, and Joji Jidoi[2]

The H_2O_2-Fe-mediated halogenation system has a potent antimicrobial activity ([4, 9, 10]) against intracellular parasites, including mycobacteria ([13]), and it is thought to play an important role in the expression of antimicrobial activity of phagocytes. *Mycobacterium leprae*, an intracellular parasite, resists the bactericidal mechanisms of phagocytes. It is impossible to estimate the number of live organisms of *M. leprae* by colony counting, because *M. leprae* cannot be grown *in vitro* on any known media. It has been reported that fluorescein diacetate-ethidium bromide (FDA/EB) staining is useful for determining the viability of mycobacterial cells, including *M. leprae* ([12]). This method is based on the following principle: FDA, a nonpolar and nonfluorescent fatty-acid ester, is transferred into intact cells through the cell membrane by an active transport system and is then hydrolyzed by cytoplasmic esterases, yielding polar fluorescent fluorescein which emits green light in response to excitation by ultraviolet light. Thus, viable cells are stained green. Ethidium bromide, on the other hand, can easily penetrate into dead cells through the damaged cell membrane but it is excluded by viable cells. EB is tightly bound to double-stranded DNA. This results in an orange fluorescence of dead cells ([2, 11]).

We examined the susceptibility of *M. leprae* and a member of the *M. avium* complex (MAC) to the microbicidal activity of the halogenation system, with or without antimocrobial agents, using FDA/EB staining.

MATERIALS AND METHODS

Organisms. *M. leprae* Thai 53 was harvested from the foot pad tissue of nude mice infected with *M. leprae* obtained from K. Kohsaka, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. The *M. leprae*-infected foot pad tissue was minced with scissors and homogenized with a glass homogenizer in Hanks' balanced salt solution (HBSS) with 5% fetal bovine serum (FBS). This homogenate was centrifuged at 100 × *g* × 5 min and four fifths of the supernate was again centrifuged at 400 × *g* × 20 min. The pellet was suspended in HBSS with 5% FBS and centrifuged at 100 × *g* × 5 min. Three fifths of the supernate was diluted with 0.15 M saline to the appropriate concentration. The strain of the *M. avium* complex, ATCC 13950, supplied by A. Y. Tsang (National Jewish Hospital and Research Center, Denver, Colorado, U.S.A.) was cultivated in Dubos' Tween®-albumin medium (Eiken Chemical Co., Tokyo, Japan) at 37°C for 7 days. The organisms were harvested by centrifugation (500 × *g* × 15 min), washed twice with 0.15 M saline, and resuspended in saline.

Chemicals. Fluorescein diacetate (FDA) and ethidium bromide (EB) were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. The antimicrobial agents used were: isonicotinic acid hydrazide (isoniazid, INH) (Wako Pure Chemical Co., Osaka, Japan); *p*-amino-salicylic acid (PAS) (Wako); 4,4'-diaminodiphenylsulfone (dapsone, DDS) (Nakarai Chemical Co., Kyoto, Japan); rifampin (RFP) (Daiichi Pharmaceutical Co., Tokyo, Japan); ofloxacin (OFLX) (Daiichi); cefoxitin (CFX) (Daiichi); clofazimine (CLO) (CIBA-GEIGY Co., Hyogo, Japan); and cofetatan (CTT) (Yamanouchi Pharmaceutical Co., Tokyo, Japan).

Antimycobacterial activity of the halogenation system. The microbicidal activity

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[2] H. Tomioka, Ph.D.; H. Saito, M.D., Ph.D., Department of Microbiology and Immunology; Y. Yamada, M.D., Ph.D.; J. Jidoi, M.D., Ph.D., Department of Dermatology, Shimane Medical University, Izumo 693, Japan.

TABLE 1. *Effect of the halogenation system, with or without antileprosy agents, on the intactness of* M. leprae *determined on a basis of FDA/EB staining.*[a]

Substrates[b] (μM)	Additions[c] (100 μM)	Percentage of green-stained cells[d]
0	—	50.5 ± 2.1
100	—	50.0 ± 1.5
0	RFP	38.4 ± 2.0
100	RFP	40.1 ± 2.5
0	DDS	53.4 ± 3.2
100	DDS	33.8 ± 0.8
0	CLO	44.0 ± 3.7
100	CLO	11.0 ± 1.0

[a] The organisms were treated by the halogenation system with or without indicated agents at 37°C for 60 min and stained with FDA/EB to determine the percentage of the green-stained cells as a parameter of intact cells.

[b] The halogenation system consists of various concentrations of substrates (sodium iodide, hydrogen peroxide, ferrous sulfate) and 20 mM acetate buffer (pH 5.5).

[c] RFP = rifampin; DDS = dapsone; CLO = clofazimine.

[d] Mean ± S.E.M. (two separate incubations).

TABLE 2. *Effect of the halogenation system, with or without antimycobacterial agents, on the intactness of* M. leprae *determined on a basis of FDA/EB staining.*[a]

Substrates (μM)	Additions[b] (100 μM)	Percentage of green-stained cells
0	—	50.8 ± 0.8
100	—	52.4 ± 4.2
0	PAS	51.5 ± 0.1
100	PAS	53.1 ± 4.9
0	INH	48.9 ± 1.9
100	INH	52.1 ± 1.7

[a] See footnotes to Table 1.

[b] PAS = *p*-amino-salicylic acid; INH = isonicotinic acid hydrazide.

of the halogenation system was measured as described previously ([13]). Briefly, the reaction mixture (2 ml), consisting of 100 μM sodium iodide, 100 μM hydrogen peroxide, 100 μM ferrous sulfate, various concentrations of antimicrobial agents, 20 mM acetate buffer (pH 5.5) and test organisms (at a final concentration of 1×10^7) organisms/ml), was incubated in a shaking water bath at 37°C for 60 min. The viability of the *M. leprae* in the resulting incubation mixture was determined by FDA/EB staining. In the case of the *M. avium* complex strain, the number of residual colony-forming units (CFU) in the incubation mixture was estimated by plating on Middlebrook 7H10 agar plates (Difco Laboratories, Detroit, Michigan, U.S.A.).

FDA/EB staining. FDA/EB staining was done by the method of Tsukiyama ([12]). Briefly, the reaction mixture was diluted with 4 ml of phosphate buffered saline (PBS) (pH 7.2) after incubation and centrifuged at 1000 × *g* × 30 min. The organisms were suspended in 0.1 ml of Dubos' Tween®-albumin medium and smeared on a glass slide. The FDA/EB solution consisted of 0.5 ml of 1 mg/ml FDA in acetone, 0.02 ml of 2 mg/ml EB in PBS (pH 6.5) containing 0.05% Tween® 80 (PBS-Tween® 80) and 4.5 ml of PBS-Tween® 80. After the smear had been incubated in the FDA/EB solution at 37°C for 60 min, the numbers of green- and orange-stained cells were counted at a magnification of 400 under a fluorescent microscope. The percentage of green-stained cells in the total cells (green- plus orange-stained cells) was then calculated.

RESULTS

Susceptibility of *M. leprae* to the halogenation system with or without antimicrobial agents. Table 1 shows the changes in the viability of *M. leprae* exposed to the halogenation system, with or without the addition of antileprosy agents, as determined by FDA/EB staining. The percentage of green-stained cells was not affected by the halogenation system alone but was reduced by the system combined with either RFP, DDS, or CLO. It is noted that RFP alone reduced the viability of the organisms. The synergistic effect was highest with CLO, followed by DDS. In cases of the antituberculosis drugs (Table 2) such as PAS and INH, there was no such decrease. As shown in Table 3, neither CFX nor CTT caused a significant decrease in green-stained cells, even in combination with the halogenation system; the halogenation system plus OFLX caused a marked reduction in the viability of the organisms.

Susceptibility of *M. avium* complex to the halogenation system. Table 4 shows the susceptibility of the MAC strain to the halogenation system on the basis of changes in viable units and in the ratio of green-stained

TABLE 3. *Effect of the halogenation system, with or without antibacterial agents, on the intactness of* M. leprae, *determined on a basis of FDA/EB staining.*[a]

Substrates (μM)	Additions[b] (100 μM)	Percentage of green-stained cells
0	—	55.2 ± 0.2
100	—	52.2 ± 1.5
0	CFX	53.6 ± 2.2
100	CFX	54.1 ± 0.6
0	CTT	52.8 ± 1.1
100	CTT	54.0 ± 2.5
0	OFLX	55.6 ± 3.1
100	OFLX	35.9 ± 1.8

[a] See footnotes to Table 1.
[b] CFX = cefoxitin; CTT = cefotetan; OFLX = ofloxacin.

cells after FDA/EB staining. The halogenation system did not affect the behavior of the organisms in FDA/EB staining, but markedly reduced the number of residual CFU. This indicates that, in the case of the MAC strain, the ability of the organisms to be stained green by FDA/EB did not correlate with the ability to grow on 7H10 agar medium. Table 5 shows the effects of the halogenation system supplemented with OFLX on the green-fluorescence intensity of the MAC strain. Even in the presence of OFLX, the halogenation system failed to reduce the number of the green-stained organisms by FDA/EB staining.

TABLE 4. *Relationship between changes in membrane intactness determined by FDA/EB staining and reduction in viable units by treatment of a MAC strain with the halogenation system.*[a]

Substrates (μM)	Percentage of green-stained cells	log CFU/ml
0	98.7 ± 1.3	7.13 ± 0.27
10	97.3 ± 1.5	7.48 ± 0.07
50	97.5 ± 0	6.43 ± 0.15
100	96.7 ± 1.3	0[b]

[a] The organisms were incubated in the halogenation system consisting of various concentrations of substrates (hydrogen peroxide, ferrous sulfate and sodium iodide) and 20 mM acetate buffer (pH 5.5) at 37°C for 60 min, and subjected to FDA/EB staining and CFU-counting on 7H10 agar plates. Other details are as in Table 1.
[b] Killed.

TABLE 5. *Effect of the halogenation system, with or without ofloxacin, on the intactness of an* M. avium *complex strain determined on a basis of FDA/EB staining.*[a]

Substrates (μM)	Ofloxacin (μg/ml)	Percentage of green-stained cells
0	0	97.2 ± 0.9
0	100	99.2 ± 0.3
100	0	98.6 ± 1.0
100	25	99.2 ± 0.1
100	100	100.0 ± 0

[a] See footnotes to Table 1.

DISCUSSION

The peroxidase- or Fe-mediated halogenation system has potent bactericidal activity ([4, 9, 10, 13]). This antimicrobial mechanism seems to be mediated by iodination and oxidation of cellular components requisite for viability, such as membrane-proteins and iron sulfur centers of the electron transport system ([4, 9, 10]). FDA is taken up by cells due to an active transport system of the cell membrane and subsequently is hydrolyzed by intracellular esterases, giving a green fluorescence. EB enters cells through the damaged cell membrane and produces an orange fluorescence by binding to DNA. These events lead to a reduction of green fluorescence and an increase in orange photoemission in the case of a damaged cell membrane ([12]). Here, we found that the H_2O_2-Fe-mediated halogenation system alone did not reduce the green-fluorescence intensity of *M. leprae* or the MAC strain. Therefore, it seems that the halogenation system alone failed to cause damage to the cell membrane of *M. leprae*, at least not to a degree that resulted in the reduction of its green fluorescence.

On the other hand, when the halogenation system was supplemented with OFLX, so-treated *M. leprae* showed a decrease in the number of green-stained cells in FDA/EB staining. OFLX primarily inhibits the supercoiling activity of DNA gyrase, and thus it does not directly cause any cell membrane damage ([3]). Therefore, it is thought that the halogenation system, even combined with OFLX, could not damage the cell membrane structure. However, it is still possible that the antimicrobial system might inactivate the active transport system of the cell

membrane in an indirect manner, causing reduction of the green fluorescence of treated organisms under FDA/EB staining.

DDS and CLO, which do not have a primary action of the cell membrane ([1, 7, 8]), also caused a decrease in the number of green-stained *M. leprae* when used in combination with the halogenation system. The mechanisms of the cell membrane function-disintegration action of these agents may be similar to that of OFLX. RFP alone reduced the percentage of green-stained cells. However, RFP is known to primarily inhibit the RNA polymerase of organisms ([6]). In studies using an electron microscope, damage to the structure of the cytoplasm, ribosomes and mesosome of the organisms by RFP was observed, but structures of the nucleus, cytoplasmic membrane and cell wall were preserved ([5]). Thus, while RFP does not cause structural damage to the cell membrane, it is possible that the integrity of the cell membrane of the organism might be reduced when protein synthesis is inhibited by RFP.

In the case of the MAC strain, the number of viable units was decreased by the halogenation system but the green-fluroescence intensity of the organisms in FDA/EB staining was not affected, even when the halogenation system was combined with OFLX. This implies that the halogenation system may inactive certain cell components essential for growth of the organisms without damaging the active transport activity or the integrity of the cell membrane. These results suggest that the functional state of the *M. leprae* cell membrane is much more susceptible to damaging action of the halogenation system supplemented with OFLX than is the MAC strain cell membrane. If such is the case, the structural and functional stability of the *M. leprae* cell membrane, including the active transport system, is thought to be much less than that of the MAC strain cell membrane.

In any case, this study revealed that a reduction of the green fluorescence of *M. leprae* cells in FDA/EB staining was produced when *M. leprae* cells were treated with the Fe-mediated halogenation system combined with an anti-*M. leprae* agent. This sytem may be useful for *in vitro* screening of antileprosy agents.

SUMMARY

The susceptibilities of *Mycobacterium leprae* and *M. avium* complex (MAC) to the H_2O_2-Fe-mediated halogenation system supplemented with antimicrobial agents were evaluated by fluorescein diacetate-ethidium bromide (FDA/EB) staining. In the case of *M. leprae*, the number of green-stained bacteria (intact cells) was reduced in the presence of the H_2O_2-Fe-mediated halogenation system supplemented with agents possessing antileprosy activity, such as rifampin, 4,4′-diaminodiphenylsulfone (dapsone), clofazimine, and ofloxacin. In the case of the MAC strain, although viable units of the organisms were reduced by the halogenation system alone, the number of green-stained cells in the FDA/EB stain was not reduced, even when the halogenation system was used in combination with ofloxacin. Because stainability of the cells is related to structural and functional intactness of the membrane, differences between *M. leprae* and the MAC strain imply possible differences in the rigidity of the cell membrane.

RESUMEN

Utilizando la tinción con diacetato de fluoresceína-bromuro de etidio (DAF/BE) se evaluó la susceptibilidad del *Mycobacterium leprae* y del complejo del *M. avium* (MAC) a la halogenación mediada por el sistema H_2O_2-Fe suplementado con agentes antimicrobianos. En el caso del *M. leprae,* el número de bacterias teñidas de verde (células intactas) se rejudo en presencia del sistema de halogenación mediado por el H_2O_2-Fe suplementado con agentes antileprosos tales como rifampina, 4, 4′-diaminodifensilsulfona (dapsona), clofazimina y ofloxacina. En el caso del complejo MAC, aunque las unidades viables de los organismos disminuyeron en presencia solo del sistema de halogenación, el número de células teñidas de verde (tinción DAF/BE) no se redujo ni siquiera cuando el sistema de halogenación se usó en combinación con la ofloxacina. Debido a que las características tintoriales de las células están relacionadas con la integridad estructural y funcional de la membrana, las diferencias entre *M. leprae* y MAC, implican posibles diferencias en la rigidez de la membrana celular.

RÉSUMÉ

On a évalué par une méthode de coloration par le diacétate-éthidium bromure de fluoresceine (FDA-EB), les susceptibilités respectives de *Mycobacterium leprae* et du complexe *M. avium* (MAC), au système d'halogénation par peroxyde d'oxygène et fer interposés, au-

quel on avait ajouté des agents microbicides. Dans le cas de *M. leprae,* le nombre de bactéries colorées en vert (c'est-à-dire de cellules intactes), était réduit en présence du système d'halogénation décrit cidessus, lorsqu'on y avait ajouté des agents ayant une activité anti-lépreuse, tels que la rifampine, la 4,4′,-diamino-diphényl-sulfone (dapsone), la clofazimine, et l'ofloxacine. Dans le cas du complexe de MAC, malgré le fait que le nombre d'unités viables de ces organismes était réduit par le système d'halogénation utilisé seul, le nombre de cellules colorées en vert par le colorant FDA/EB n'était pas diminué, et ce en dépit du fait que le système d'halogénation était utilisé en combinaison avec l'oflaxacine. La colorabilité des cellules étant influrencée par l'intégrité structurelle et fonctionnelle de la membrane, les différences notées entre *M. leprae* et le complexe de *M. avium* dans cette étude suggèrent que la rigidité des membranes cellulaires de ces organismes pourrait présenter des différences.

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