THE RELATIONSHIP BETWEEN THE CHEMICAL STRUCTURE OF FATTY ACIDS AND THEIR MYCOBACTERICIDAL ACTIVITY*

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(Received: February 15, 1977)

SUMMARY: The bactericidal activity of long-chain fatty acids on mycobacteria was examined by exposing the organisms to these acids at 0.04 mM in 0.05 M acetate buffer (pH 5.6). The lethal effect of saturated fatty acids was related to the chain-length of hydrocarbon, C₁₄:₀ being the strongest in the activity and longer and shorter fatty acids being less active. Unsaturation, isomerism and the presence of α-hydroxy group were found to be other factors governing the activity. The lethal effect was greater in the order of C₁₈:₃ > C₁₈:₂ > C₁₈:₁ (cis) > C₁₈:₁ (trans) > α-OH C₁₈:₀ > C₁₈:₀. C₂₀:₄ was placed between C₁₈:₃ and C₁₈:₂ in this respect. Esterification of C₁₄:₀, C₁₈:₁ and C₂₀:₄ to methyl esters and cholesteryl esters abolished completely the bactericidal activity of these acids, suggesting the requirement of carboxyl group for the activity. The relationship between the fatty acid structure and the lethal effect was discussed in reference to these observations.

INTRODUCTION

In the previous two papers (Kondo and Kanai, 1972, 1976), we reported that some fatty acids of free form in trace amounts were highly toxic to mycobacteria, probably acting on the cytoplasmic membrane. The lethal effect was found to be related to the chain-length of hydrocarbon, the degree of unsaturation, pH, temperature and the amount of test bacilli. The present experiments were undertaken to expand our observations to more variety of fatty acids. The results allowed us to suggest some relationship between their chemical structure and the bactericidal effect.

MATERIALS AND METHODS

Micro-organisms: A highly virulent strain (Ravenel) of Mycobacterium bovis and an avirulent strain (H₃₇Ra) of M. tuberculosis were used as test organisms. These strains have been maintained on Sauton synthetic liquid medium by subculture, and aqueous suspensions of the organisms were prepared by the method described elsewhere (Kanai and Kondo, 1971).

* This study was partly supported by a grant from the Ministry of Education.
Fatty acids: Acetic (C₂:0), butyric (C₄:0), caproic (C₆:0), caprilic (C₈:0), capric (C₁₀:0), Lauric (C₁₂:0), myristic (C₁₄:0), palmitic (C₁₆:0), stearic (C₁₈:0), and arachidic (C₂₀:0) acids were the saturated fatty acids employed. As unsaturated acids, oleic acid (cis Δ⁹ C₁₈:1), elaidic (trans Δ⁹ C₁₈:1), cis and trans vaccenic acids (Δ¹¹ C₁₈:1), linoleic acid (C₁₈:2), linolenic acid (C₁₈:3) and arachidonic acid (Δ₁₂₀:₄) were used. α-Hydroxy palmitic, α-hydroxy stearic, and 12-hydroxy stearic acids were examined in some tests. Methyl esters of C₁₄:0, C₁₈:1, and C₂₀:₄, and cholesteryl esters of C₁₈:1 and C₂₀:₄ were also employed for comparison with the forms. The hydroxy fatty acids were provided by Dr. Nagai, Tokyo Metropolitan Institute of Gerontology. The rest of the compounds were of approximately 99% purity (Sigma).

Exposure system: An acetate buffer solution (0.05 M, pH 5.6) was dispensed in amounts of 4.5 ml into test tubes which had been washed with detergent, treated with a potassium dichromate-sulfuric acid solution, and rinsed well to be freed from dust and fatty acids. A stock acetone solution of fatty acids (2 mM) was added to the tubes in amounts of 0.1 ml. The mixtures were gradually heated in a water-bath to achieve sterilization and acetone evaporation. The solutions or slightly opalescent suspensions of fatty acids thus obtained were then mixed with 0.5-ml portions of a mycobacterial suspension. The mixtures were incubated in a water-bath at 37 °C for a given period.

Estimation of mycobactericidal effect: After incubation, a portion of the fatty acid-mycobacteria mixture was subjected to serial 10-fold dilutions down to 10⁻⁶ mg bacilli per ml; 0.1-ml portions of appropriate dilutions were inoculated onto slants of Ogawa glycerol-egg medium. The number of colonies developed after 4 weeks of incubation allowed calculation of viable units of the original mixture.

**Results**

In the incubation mixture, the final concentration of fatty acid was 0.04 mM. Among the saturated fatty acids, C₁₄:0 was most active in the bactericidal effect on *M. bovis* (Ravenel). The rate of reduction in viable units was almost 1/10,000 in 2 hr (Fig. 1). C₁₆:0 was the second strongest in the activity, but the other fatty acids were without effect at this concentration. This result was different from our previous experience that C₁₂:0 was much more effective than C₁₆:0 in the same concentration of 20 μg/ml, the former being lower in molar concentration.

The same type of experiment was repeated with six saturated fatty acids from C₈:0 to C₁₈:0 and three unsaturated fatty acids C₁₈:1, C₁₈:2, and C₂₀:₄, in which a virulent strain (Ravenel) of *M. bovis* and an avirulent strain (H₃₇Ra) of *M. tuberculosis* were compared. As shown in Fig. 2, the high lethal effect of C₁₄:0 was confirmed, but even stronger activity was demonstrated with the unsaturated fatty acids. In this respect, there was no difference between the two
Fig. 1. Viable counts of a suspension of *M. bovis* (Ravenel) exposed to saturated fatty acids (0.04 mM) at pH 5.6 and 37°C for 2 hr.

Fig. 2. Viable counts of suspension of virulent *M. bovis* (Ravenel) and avirulent *M. tuberculosis* (H37Ra) exposed to saturated or unsaturated long-chain fatty acids (0.04 mM) at pH 5.6 and 37°C for 1 hr.
Fig. 3. Viable counts of a suspension of *M. bovis* (Ravenel) exposed to bactericidal long-chain fatty acids (0.04 mM of C14:0, C18:1 and C20:4) and their methyl- and cholesteryl esters at pH 5.6 and 37°C for 1 hr.

Fig. 4. Lethal effects of C18:0 and C20:0 fatty acids in response to the number of double bond on *M. bovis* (Ravenel).

The bacterial suspension was exposed to a given fatty acid at a concentration of 0.04 mM at pH 5.6 and 37°C.
strains. However, additional experiments confirming the effectiveness of C₁₄:₀, C₁₈:₁ and C₂₀:₄ together with the ineffectiveness of their methyl and cholesteryl esters revealed that the esterification abolished completely the killing effect of the fatty acids (Fig. 3).

In order to grade the lethal effects of unsaturated fatty acids, a time-course observation was made by following the change in viable counts of *M. bovis* (Ravenel) exposed to 0.04 mM for 10, 20 or 30 min. Figure 4 shows that the activity was strengthened by the increase of the double-bonding in C₁₈ fatty acid. C₁₈:₃ was most active, and viability of the suspension was reduced thereby down to an undetectable level in as early as 10 min. It is noteworthy that C₁₈:₃ was more effective than C₂₀:₄.

Figure 5 shows the result of an experiment designed to see the relation between isomerism and the lethal effect of C₁₈:₁ fatty acid. The time-course observation of viable counts of *M. bovis* (Ravenel) exposed to 0.04 mM demonstrated that cis form is more effective than trans form. A similar type of experiment was conducted to see the effect of hydroxy group in α- and 12-position on the activity of C₁₈:₀ or C₁₆:₀ fatty acid. The results are shown in Fig. 6.

![Fig. 5. Comparison between cis and trans forms of C₁₈:₁ fatty acids.](image)

The bacterial suspension was exposed to a given fatty acid at a concentration of 0.04 mM at pH 5.6 and 37 C.
Fig. 6. Increased lethal effects on M. bovis (Ravenel) of C_{18} fatty acid having a hydroxy group in \( \alpha \)-position, but not in 12-position.

The bacterial suspension was exposed to a given acid at a concentration of 0.04 mM at pH 5.6 and 37° C.

The presence of \( \alpha \)-OH, but not 12-OH, gave the bactericidal activity to essentially inactive C_{18:0} fatty acid. With C_{16:0}, the same tendency was noted, though not so remarkable as with C_{18:0}.

**DISCUSSION**

The lethal effect of long-chain fatty acids on mycobacteria will be discussed assuming the presence of two stages in the bacillus-fatty acid association. The first stage must be the adsorption of fatty acid on the bacterial surface. This would be governed by the physicochemical properties of the acids in solution and those of the cell surface of the organisms. Long-chain fatty acids must be in solution to express their antibacterial activity, but they must still be lipophilic to be adsorbed onto the cell surface. In this regard, the optimum polar and nonpolar structure of the saturated fatty acid molecules might be myristic acid for highly lipophilic mycobacteria. However, in the case of more hydrophilic...
gram-positive bacteria, e.g. *Staphylococcus aureus*, lauric acid was more effective than myristic acid (Galbraith et al., 1971; Kondo and Kanai, unpublished data).

The increased lethal effect of C₁₈:₀ by polyunsaturation would be also explained by the fact that such unsaturated fatty acids behave as liquids in solution instead of as hydrated solids and thus combining increased solubility with high lipophilic activity (Galbraith et al., 1971).

The second stage might be concerned with the association of fatty acids with the cytoplasmic membrane, since it was suggested by Kondo and Kanai (1976) that the site of action on mycobacteria is the membrane just like in other gram-positive bacteria (Galbraith and Miller, 1973). It has been postulated that fatty acids insert their nonpolar radicals into the phospholipid layer of the biomembrane thus increasing its negative charge. This may result in increased permeability of the membrane. In such a situation, the surface-area requirements of fatty acid molecules will be an important factor for their effects. The presence of double bonds and cis forms rather than trans forms would increase the surface area of fatty acids (Netter, 1969), and the greater is the area occupied by an individual molecule the fewer molecules will be required to occupy the sensitive site in the bacterial membrane. This rationale would be able to explain the order of effectiveness of C₁₈ fatty acids, C₁₈:₃ > C₁₈:₂ > C₁₈:₁ (cis) > C₁₈:₁ (trans) > C₁₈:₀. Interestingly enough, this is also the order of their melting point from low to high.

Likewise, the appearance of the lethal effect in C₁₈:₀ by addition of a hydroxy group in α-position might be due to the increased solubility and the steric effect of its particular OH location. The necessity for a polar end group in the killing process was demonstrated by the observation that their methyl esters and cholesteryl esters were quite inactive. Fay and Farias (1976) and Kodicek (1949) have already pointed out this fact, though no direct correlation between surfactant and antibacterial activity was noted by the latter author.

Further studies are now in progress to examine whether or not long-chain fatty acids can have an opportunity to manifest their mycobactericidal activity in the host-tissue environment.

**References**


