Letters to the Editor

Neutral Red Staining of Cells of a Sulfolipid-Deficient *Mycobacterium tuberculosis* *pks2* Mutant Proves that Sulfolipids Are Not Responsible for This Cytochemical Reaction

In 1948 Dubos and Middlebrook reported that the cells of the virulent H37Rv *Mycobacterium tuberculosis* strain were able to reduce neutral red and become red in color (NR⁺), whereas the cells of the avirulent H37Ra *M. tuberculosis* were not (NR⁻) (3, 7). During the following decade, neutral red staining was performed on *M. tuberculosis* clinically isolated strains, *Mycobacterium bovis*, and environmental mycobacteria (1, 4–6). Recently, we have also performed this staining in *M. tuberculosis* strains (11). The different studies showed a concordance of results; that is, cells of the virulent *M. tuberculosis* and *M. bovis* strains stained red, while cells of the majority of environmental mycobacteria remained unstained.

In 1959 Middlebrook and coworkers studied the cellular components responsible for neutral red staining and established a positive correlation between the sulfolipid content and the neutral-red-staining cells (8). Although the results of this single work were not conclusive, the idea that sulfolipids are responsible for the NR⁺ cells of the *M. tuberculosis* virulent strains has been widely quoted and accepted. However, no direct evidence has confirmed this conclusion.

Recently, a *pks2* mutant of *M. tuberculosis* H37Rv has been generated that is unable to produce hepta- and octamethyl phthioceranic acids, the major acyl constituents of sulfolipids, and is thus unable to produce sulfolipids (10). The availability of this mutant made it possible to test directly whether sulfolipids are responsible for neutral red staining.

The *M. tuberculosis* strains used in this study were the two

reference strains H37Rv (ATCC 27294) and H37Ra (ATCC 25177), the H37Rv *pks2* mutant, and the H37Rv *msl3* mutant (2). This last mutant is able to produce sulfolipids but is deficient in polyacyl and diacyl trehaloses and has been used as a control together with the H37Rv wild-type and H37Ra strains.

The neutral red staining was performed in a test tube as described previously (11). The strains were tested several times, and the results shown in Fig. 1 were obtained each time. As shown in Fig. 1, cells of the *pks2* mutant were NR⁺, as were cells of the wild-type H37Rv strain and the *msl3* mutant. H37Ra cells were NR⁻. Consequently, we can conclude that sulfolipids are not responsible for the ability of the cells of virulent *M. tuberculosis* strains to become red stained.

In the past a correlation was accepted between neutral red staining and virulence in *M. tuberculosis*, and as sulfolipids were thought to be responsible for this reaction, they were considered a virulence factor until recently. However, a new study has shown that the *M. tuberculosis pks2* sulfolipid-deficient mutant showed the same degree of virulence as the wild type (9). The present finding that sulfolipids are not involved in neutral red staining is consistent with this result. The mycobacterial compound(s) responsible for neutral red staining in *M. tuberculosis*, with a possible correlation to virulence, remains to be identified.

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FIG. 1. Neutral red staining in a test tube. From left to right, the *M. tuberculosis* strains are *pks2*, *msl3*, H37Ra, and H37Rv.

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