Mycosides C: Behavior as Receptor Site Substance for Mycobacteriophage D4

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Interpretation of an earlier published infrared spectrum of *Mycobacterium smegmatis* lipids with receptor site activity for D4 phage led us to the inference that the active substance is very likely a mycoside C. This hypothesis was confirmed: the well-characterized mycosides C_s and C₁₂₁₇ elaborated by the heterologous strains *M. scrofulaceum* and *Mycobacterium* species 1217, respectively, are essentially indistinguishable from the *smegmatis* lipids in their behavior toward D4. Minute quantities adsorb and extensively inactivate the phage on appropriate incubations. In accord with derivative expectations, *Mycobacterium* species 1217 is a permissive host, attacked and lysed by D4. However, our current strains of *M. butyricum*, *M. avium*, and *M. scrofulaceum*, which reputedly produce various related mycosides C, are neither lysed by nor do they significantly adsorb the phage. Implications of these observations are discussed.

Mycobacterial lipids have been implicated in mycobacteriophage-host interactions—particularly as receptor site substances for the phage. Convincing evidence derives from studies in which mycobacterial lipids demonstrably adsorbed specific mycobacteriophage and thus rendered them inactive toward the host (2, 4). The behavior of active substances in adsorption-inactivation phenomena shares many features in common with the adsorption-infection process involving intact host cells: antagonism by Tween, a requirement for certain levels of Ca²⁺, triggering release of phage deoxyribonucleic acid, and specificity (11)—observations which clearly support the putative role of the specific lipids as receptor site substances.

In a series of studies, Tokunaga et al. (10, 11) obtained lipid extracts of *Mycobacterium smegmatis* ATCC 607 which extensively inactivated D29 and D4 phage by adsorbing these during appropriate incubations. Two partially purified substances, exhibiting specific activity against the individual phages, were separated. From color reactions on thin-layer chromatograms of the most active fractions obtained by silicic acid chromatography, and from an infrared spectrum of the partly purified lipid, Tokunaga et al. (9) tentatively concluded that the receptor site substance for D4 was a "lipid containing sugar."

A combination of circumstances led us to

recognize the resemblance between the above-mentioned infrared spectrum and spectra of the mycobacterial *peptidoglycolipids*, designated as mycosides C, and thus to the inference that these peripherally located substances (3, 5, 6) might have the receptor function. Chemical structures of some half dozen of these lipids have been elucidated. The simpler examples have a generalized structure (Fig. 1). The "N-variable" portion and the allo-threonine are ordinarily (glycosidically) linked to a methylated rhamnose and an acetylated 6-deoxytalose, respectively. Variations in the acyl function are common and permute with the variety of sugars to yield the spectrum of C-mycosides which have been described.

The present studies indicate that the well-characterized mycosides C₁₂₁₇ and C_s derived, respectively, from the *heterologous* strains *Mycobacterium* species 1217 and *M. scrofulaceum* behave toward D4 like the lipid receptor site substances extracted from *M. smegmatis* ATCC 607; coupled with additional physical evidence, these findings support the conclusion that the latter are quite probably mycosides C. [These results were reported at the Sixth Joint Conference, Tuberculosis Panel, United States-Japan Joint Cooperative Medical Science Program (San Francisco, October 1971) and were confirmed at the same session by T. Murohashi: in studies which very likely antedated our own, Tokunaga, Furuchi, and Murohashi independently concluded that the D4 receptor substance from ATCC 607

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tion-inactivation experiments, 0.1 ml of lipid suspensions containing 2 or 10 μg of lipid were mixed with 0.8 ml of HIB and then with 0.1 ml of phage suspension (5×10^6 PFU). After incubation at 37 C for 30 min, dilutions of the mixtures were plated on *M. smegmatis* ATCC 607. As controls, phage were incubated in HIB alone as well as in HIB containing aqueous dioxane equivalent to that added with the lipid suspensions. No inactivation due to the modest dioxane levels was encountered. Activities with respect to both D4 and D29 were examined. The results are summarized graphically in Fig. 2 and confirm that the *heterologous* mycosides C_{1217} , $C_{1217}\text{-A}$, and C_s behave like the active lipids derived from extraction of the host *M. smegmatis* in adsorbing and inactivating D4; they do not, however, have activity with respect to D29 (under the same conditions in which adsorption-inactivation was expressed for D4, neither mycoside C_s nor C_{1217} at even 20- μg levels effected any inactivation of phage D29).

Electron microscopy. Examination of samples prepared as described (methods) showed that the ordinarily amorphous mycoside particles (*not shown*) actively adsorbed numerous phage, often with concomitant triggering of deoxyribonucleic acid release, as suggested by the plethora of ghosts. A "brush-heap" of microcrystalline mycoside Csm-3 is illustrated in Fig. 3a. Figures 3b and c show essentially identical filamentous needles (*see above*) of mycosides Csm-3 and C_{1217} , respectively, after incubation with D4. In the absence of mycosides, the phage were sparse: they remained free, unattached to the inert control

substances—such as calcium phosphate or, as in Fig. 3d, to alkali-degraded cell wall particles of *M. tuberculosis* strain H37Rv.

Host range. Since many mycobacterial species elaborate a variety of mycosides C, we examined some of these strains for their interaction with mycobacteriophage D4. These tests indicated that individual strains at hand of *M. butyricum*, *M. scrofulaceum*, and two strains of *M. avium*—as well as *M. intracellulare* (serotype Davis) and *M. fortuitum* (not known to elaborate mycoside C)—were neither lysed by nor did they significantly adsorb D4. *Mycobacterium* species 1217, however, adsorbed the phage as effectively as ATCC 607 and served as a permissive host with about half the plaque-forming efficiency exhibited by the latter.

DISCUSSION

The infrared spectrum of Csm-3 (Fig. 4A) is nearly identical to that of mycoside $C_{1217}\text{-A}$ (Fig. 4B) as well as to the spectrum of active extracts described by Tokunaga et al. (9). These spectra reflect the peptidoglycolipid character of the mycosides C and are in accord with the gross structure presumed for the D4 receptor substance of *M. smegmatis* ATCC 607: absorption (arrows) at about 3,300, 1,640, and 1,540 cm^{-1} attributable to the peptide; ester absorption (1,740 cm^{-1}), specifically associated in the mycosides C with acetate substituents (1,245 cm^{-1}) in the carbohydrate residues. In the "fingerprint" region below 1,000 cm^{-1} , the two spectra show convincing similarities. We nevertheless do not suggest that the C_{1217} and the active extract of ATCC 607 are identical. [E. Vilks has indicated that *M. smegmatis* produces a mycoside C in culture (12).]

Mycosides C_s and C_{1217} differ significantly from one another in structure, both in the fatty acid(s) and in a sugar moiety (5, 12). Thus the somewhat greater biological activity of C_{1217} and of the *smegmatis* extracts (Fig. 2) indicates an ability of the phage to distinguish at least some structural differences but does not suggest a highly restrictive selectivity. The more homogeneous $C_{1217}\text{-A}$ is apparently more active than the partially purified C_{1217} ; conversely, the "crude" Sm lipids consistently exhibited greater activity than Csm-3, suggesting that within the former we may yet discern homogeneous species with maximum specificity for phage D4. On the other hand the failure of the C-mycosides to inactivate D29 is in accord with the specificity previously observed by Tokunaga et al., who characterized the D29-specific substance as very likely one or more phospholipids (9).

The resistance of our current strains of *M. butyricum*, *M. scrofulaceum*, and *M. avium* to

FIG. 2. Inactivation of phage D4 by various mycosides C (10 and 2 μg per 5×10^6 PFU).

FIG. 3. *a*, “Brush-heap” of microcrystalline mycoside Csm-3 ($\times 35,000$); *b*, *c*, filamentous needles of Csm-3 and C₁₂₁₇ after incubation with D4 ($\times 35,000$); *d*, alkali-degraded H37Rv cell wall particles incubated with D4 ($\times 35,000$).

FIG. 4. Infrared spectra comparing: *A*, mycoside Csm-3; *B*, mycoside C₁₂₁₇-A.

infection by D4, and their apparent inability to adsorb this phage, seem curious and may be due to any of several factors. Since mycoside C_{b1} of *M. butyricum* is structurally very similar to C₁₂₁₇ (5, 13) and since we demonstrated the receptor site activity of mycoside C_s, this apparent inertness may indicate failure of our strains of *M. butyricum* and *M. scrofulaceum* to elaborate their respective mycosides C; or if these are synthesized they may not be surface constituents in these particular strains. Studies to clarify these issues are currently in progress.

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