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Calixarenes with Host-Mediated Potency in Experimental Tuberculosis: Further Evidence that Macrophage Lipids Are Involved in Their Mechanism of Action

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Some time ago, it was found that attachment of hydrophilic polyoxyethylene chains to various hydrophobic phenols and alcohols gave water-soluble products which, although inactive in vitro, influenced an experimental tuberculous infection. With short chains the infection was suppressed, and with long chains it was promoted. Later work concentrated on Macrocyclon (short chain) and HOC-60 (long chain), both derived from a hydrophobic, polyphenolic calixarene. Growth of *Mycobacterium tuberculosis* inside macrophages (Mϕ) was inhibited by Macrocyclon and stimulated by HOC-60. Also, triglyceride lipase from Mϕ extracts and an extracellular phospholipase were inhibited by Macrocyclon and stimulated by HOC-60. This suggestion of a mechanism has been strengthened by the finding that Mϕ cultivated in monolayers and treated with Macrocyclon showed accumulation of lipid and little formation of fatty acid after incubation of killed cells. With HOC-60, lipid was depleted and much fatty acid was found.

It was reported in 1955 (3) that attachment of hydrophilic polyoxyethylene chains to various lipophilic polyphenols, by condensation with ethylene oxide, gave water-soluble products which, although inactive against *Mycobacterium tuberculosis* in cell-free medium, had striking and prolonged effects on experimental murine tuberculosis after parenteral administration. Similar effects of the reaction products of certain alcohols (e.g., cholesterol) with ethylene oxide were later shown (4). In all such preparations, short hydrophilic chains consistently had the best antituberculous activity; with long chains, the effect was reversed and the course of disease was accelerated. Importantly, dose changes did not convert an inhibitor to an accelerator, or vice versa.

Since 1955, most of the experimental work has been done with Macrocyclon (an effective antituberculous preparation) and HOC-60 (a representative "protuberculous" preparation). These were made by condensing ethylene oxide with "HOC-phenol," a macrocyclic octylphenol-formaldehyde oligomer of the type now called calixarenes (6, 7), to give an average polyoxyethylene chain length of 12.5 (Macrocyclon) or 60 (HOC-60) ethylene oxide units. It was previously thought that HOC-phenol was a tetramer (3, 4), but subsequent work by Gutsche (6, 7) on this class of compounds indicated an octamer. Thus, Macrocyclon and HOC-60 have mean molecular weights of around 6,200 and 23,000, respectively. They appear to be virtually nontoxic in experimental use.

Macrocyclon and HOC-60 enter macrophages (Mϕ) by endocytosis and are lysosomotropic (1, 8). In cultivated Mϕ infected with *M. tuberculosis*, Macrocyclon is bacteriostatic and HOC-60 accelerates bacterial growth (1, 8); from their inactivity in vitro, the effects thus seem to be host cell mediated. Exploration in various other systems failed to show similarly contrasting effects by these preparations that are related to hydrophilic-lipophilic balance (2, 5, 8), but analogous contrary effects have been observed in two disparate areas. First, antituberculous and protuberculous preparations, respectively, decreased and increased lysis of erythrocytes after sudden cooling (15). Second, antituberculous preparations inhibited, and protuberculous preparations enhanced, the in vitro activity of triacylglycerol lipase extracted from Mϕ (10) and of a phospholipase A_2 model system (12). Some causal relationship between intracellular lipid and the effects of intracellular Macrocyclon on tubercle bacilli was suggested by extracts of lysosomes isolated from livers of pretreated rats, which contained increased lipids and directly inhibited bacterial growth in vitro (9). We have investigated these clues by examining patterns of lipid distribution and lipase activity in intact Mϕ after exposure to Macrocyclon and HOC-60.

Resident peritoneal Mϕ from female mice (albino Parkes strain) were cultivated at 37°C as monolayers in a high-concentration serum medium (for long-term survival) (1, 11). The medium was changed after 2 to 6 days, and Macrocyclon or HOC-60 was included in the fresh medium in doses equivalent to those used for chemotherapy. Macrocyclon (125 mg/ml in 77 mM NaCl) was adjusted to pH 7 and autoclaved; 0.05 ml of this solution was added to 1 ml of culture medium, giving approximately 1 mM Macrocyclon; HOC-60 was treated similarly and applied at 0.25 mM (8). Controls received 0.05 ml of 0.15 M NaCl. Incubation was then continued for up to 18 days, usually without a second medium change. Mϕ monolayers became swollen in the presence of Macrocyclon or HOC-60 but remained viable as judged by response to supravital staining and capacity for ingestion of microorganisms. The effect was slowly reversible. Oil Red 4B (13) was used to stain neutral lipids (14, 18), predominantly triglycerides (16, 19). A stock solution (1.0 g/100 ml of 70% isopropanol) was shaken well and left to settle for 24 h. For use, 3 parts of this supernatant plus 2 parts of distilled water were allowed to stand for a few min until opaque and then filtered. Coverslips withdrawn from the culture tubes were washed well in 0.15 M NaCl and fixed for 30 min in filtered formal-calcium (10 ml of neutralized 40% formaldehyde, 10 ml of 10% anhydrous $CaCl_2$, distilled water to 100 ml). After being washed well in running tapwater and then distilled water, the coverslips were stained for exactly 30 min in the Oil Red solution, washed briefly in running water to remove free stain, differentiated in 60% ethanol for a few sec-

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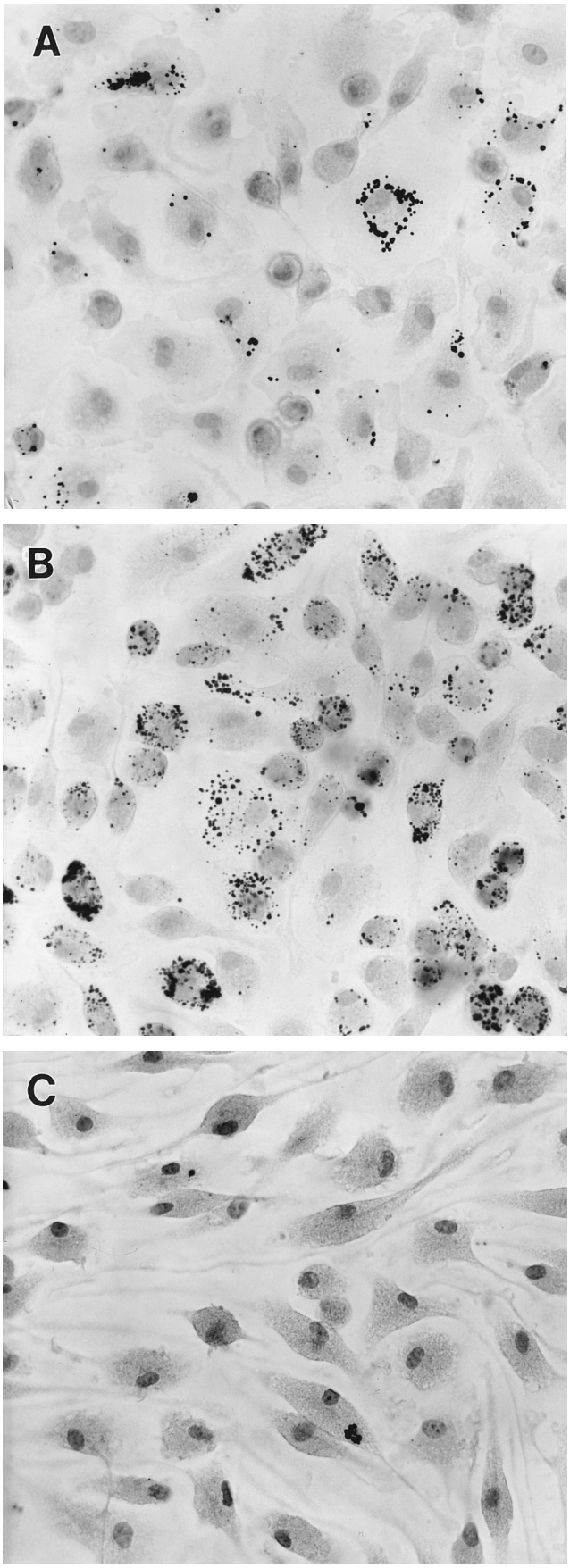

FIG. 1. Contrasting effects of representative short- and long-chain calixarenes (HOC-12.5 [Macrocyclon] and HOC-60) on Mϕ lipid. Monolayers were cultivated for 17 days in medium with or without one of the agents and then fixed and stained with Oil Red 4B. (A) Control culture medium containing no agent shows a normal appearance and distribution of stainable droplets. (B) Medium containing Macrocyclon shows accumulation of droplets. (C) Medium containing HOC-60 shows depletion of droplets. Magnification, ×420.

onds, and washed again in running tapwater for 1 to 2 min. Finally, the coverslips were counterstained with Ehrlich's hematoxylin, blued in running water, and mounted in glycerin-jelly. Assessment of lipase activity was adapted from a previously described method (17). Coverslips were withdrawn, washed in 0.15 M NaCl, and fixed in 2.5% glutaraldehyde in

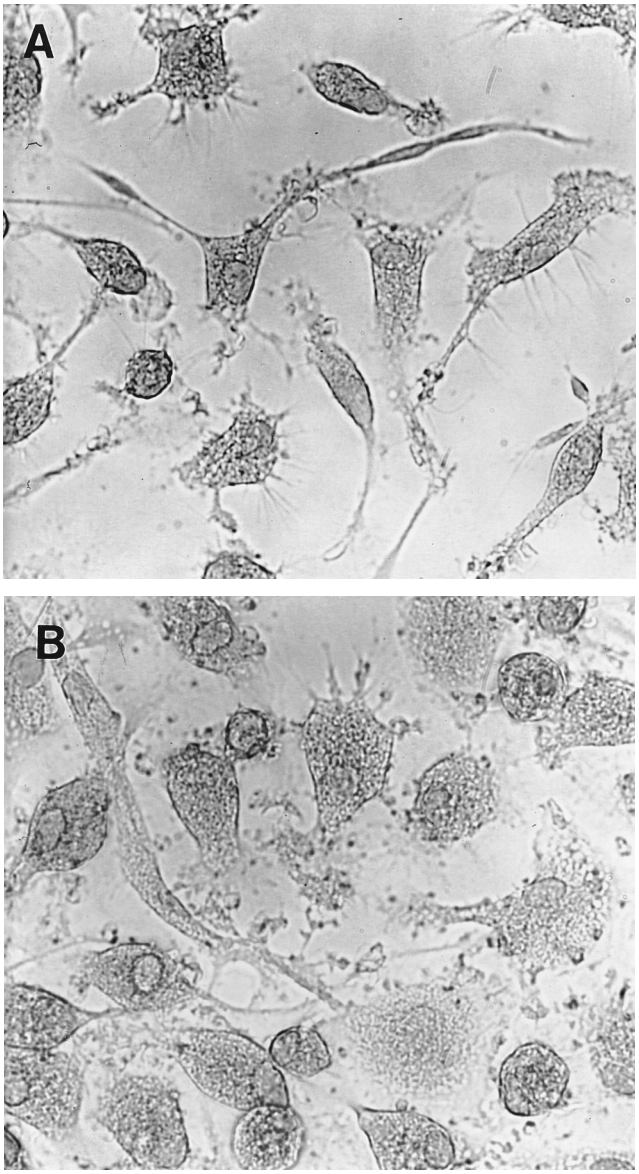

FIG. 2. Contrasting effects of the two calixarenes on Mϕ lipase activity. Monolayers were cultivated for 9 days in medium with one of the agents and then processed for lipase activity. (A) Medium containing Macrocyclon shows few lead sulfide deposits. (B) Medium containing HOC-60 shows a profusion of cell-associated lead sulfide deposits. Magnification, ×700.

sodium cacodylate buffer (pH 7.0) at 4°C for 10 min. After being washed in three changes of 0.15 M NaCl, they were incubated for 18 h at 37°C (i) in a mixture of 80 parts (by volume) of 0.05 M sodium acetate-acetic acid buffer, 10 parts of 1.5 M NaCl, and 10 parts of 0.2 M $CaCl_2$–$2H_2O$, adjusted to either pH 4.6 or pH 5.6, or (ii) in a mixture of 90 parts of 0.1 M Tris-HCl buffer and 10 parts of 0.2 M $CaCl_2$–$2H_2O$, adjusted to pH 8.6. After being further washed in 0.15 M NaCl and then water, the coverslips were immersed for 30 min at 37°C in freshly prepared 2.0% lead nitrate, washed in water, exposed for 5 min to a saturated solution of H_2S in water, washed again, and mounted. As a control procedure, some coverslips were immersed in 0.15 M NaCl at 70°C for 15 min before incubation in the acid buffer mixture.

The change of medium after 2 to 6 days caused little alteration in the form and distribution of Oil Red-stainable droplets in the cells for up to 6 days thereafter; subsequent changes were best seen 12 to 16 days after the change. Cells exposed to HOC-60 were then almost totally depleted of stainable lipid droplets, cells exposed to Macrocyclon had large numbers of predominantly small droplets, and cultures without either agent showed only a decrease in the proportion of lipid-bearing cells (Fig. 1). Cells at this stage were examined cytochemically for lipase activity. When the surfactant was HOC-60, a profusion of small cell-associated deposits of lead sulfide (associated with the presence of fatty acids) was seen. With Macrocyclon, few deposits were visible (Fig. 2). Untreated cells showed variable amounts of deposits. The deposits appeared both superficially at the membrane level and within the cells. No deposit was obtainable when the monolayers had been preheated to 70°C for 15 min and little, if any, when a pH 8.6 buffer was used for incubation.

Growth of *M. tuberculosis* inside Mϕ is inhibited by Macrocylon and stimulated by HOC-60; lipase activity within similarly treated Mϕ is inhibited by Macrocyclon and stimulated by HOC-60. This observation strengthens the evidence that lipids and lipid metabolism are involved in the interactions induced by these substances in experimental animals with *M. tuberculosis* infections, in particular in their Mϕ. If this is so, it indicates a unique mechanism, apparently different from that of all other agents against this disease, a mechanism in which the host itself plays the essential part. Since resistance to conventional chemotherapeutic agents seems to be increasing, new approaches are needed.

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