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Role of Mycobactin in the Growth and Virulence of Tubercle Bacilli

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Tubercle bacilli failed to grow in iron-void media enriched with solutions of iron-containing transferrin (Tr) or ferritin (F) because these substances do not provide the bacilli with iron, which is essential for their growth. Animal serum and macrophages possessed no iron carrier with an ability to satisfy the need of the bacteria for the metal. Mycobactin (M), the growth-product of tubercle bacilli, removed iron from Tr and F and supplied the metal for bacillary utilization. The role of M in the growth of tubercle bacilli was influenced by nonionic surfactants which inhibited bacillary growth by removing M from the bacillary cells and interfering with the absorption of M-iron complexes. Experiments with Tween 80, Triton WR-1339, and lecithin showed that avirulent bacilli lose M at lower concentrations of the surfactants than virulent bacilli. Since avirulent and virulent bacilli possess the same amount of M, these findings indicate that M is bound more firmly to lipid-rich virulent than lipid-poor avirulent cells. These findings indicate that the resistance of virulent bacilli to the M-removing activity of the surfactants is an indicator of their ability to multiply in the infected host and may be used as a measure of bacillary virulence.

During the past several years, studies in several laboratories defined the competition between animals and bacterial parasites for iron, which is needed for the growth of mammalian and bacterial cells (11, 20). We found that the growth of tubercle bacilli is inhibited in most mammalian sera because they fail to provide the parasite with growth-essential iron (10). The addition of exogenous iron or of iron-chelating bacillary product mycobactin (M) to sera alleviated tuberculostasis and induced a prolific bacillary multiplication (12). The investigation of the role of M in the neutralization of serum tuberculostasis revealed that M does not serve as a growth factor but as a carrier of growth-essential iron which M removes from transferrin (Tr) and provides to tubercle bacilli in a utilizable form (15). The role of M in the development of tuberculosis can be limited by BCG and lipopolysaccharide treatments which induce hypoferremia and increase the degree of serum tuberculostasis in treated animals (14). The consideration of induced hypoferremia led to the development of nutritional immunity concept which may play a major role in the host-parasite relationships (11).

It has been reported that infectious hypoferremia results from the shift of iron from blood to phagocytic cells of the reticuloendothelial system (7), where the metal is stored in ferritin (F) molecules. Since F has been found in close association with intracellular bacteria (2), it became necessary to determine whether iron stored in F is available for bacillary utilization. Attempts will be made in this study to assess the role of M in the survival of tubercle bacilli in the presence of F and to define this role in terms of bacillary virulence.

This paper presents a portion of a Ph.D. dissertation submitted by C. A. Golden to the Graduate Faculty of the Department of Microbiology, Miami University, Oxford, Ohio, 1973.

MATERIALS AND METHODS

Bacteria. The role of M in the survival and virulence of tubercle bacilli was studied by the use of the attenuated bovine strain (BCG) of *Mycobacterium bovis*, the avirulent human strain (H37Ra) of *M. tuberculosis*, and the virulent human strain (H37Rv) of *M. tuberculosis*. Cultures of these bacteria were maintained at 37 C in Dubos Tween-albumin dextrose medium. Bacillary inocula were prepared from 5- to 10-day-old cultures; the bacilli were harvested from the medium by centrifugation at 500 × *g*, washed twice with physiological saline solution, and resuspended in saline. The bacillary suspensions were adjusted to the desired turbidity by the use of a Klett-Summerson photoelectric colorimeter.

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Iron solutions and iron-containing compounds. Since minute amounts of iron present in water or on glassware could greatly influence the effect of M on the growth of tubercle bacilli, only double-distilled water and acid-cleaned glassware were used in this study. The role of M in bacillary survival was determined in the presence of free iron or iron-containing compounds.

M was generously provided for this study by G. A. Snow (19). Since M is poorly soluble in water, it was dissolved in 70% ethyl alcohol by vigorous mixing in a Mickle tissue disintegrator. The solution, containing 1 mg of M per 1 ml of ethyl alcohol, was diluted subsequently in saline containing 0.02% of Tween 80 (Fisher Scientific Co.) to a final concentration of 0.1 mg of M per 1 ml of the solution.

Iron in the form of ferric ammonium citrate was maintained as a stock solution which contained 0.8 mg of iron per 1 ml of saline. Precise determination of the iron concentration in the stock solution of ferric ammonium citrate as well as in other iron-containing compounds was made by the method of Landers and Zak (16). Iron-providing properties of F, Imferon, and Blutal for the growth of tubercle bacilli were determined in the presence and absence of M. Cadmium-free F (twice crystallized; Pentex Inc., Kankakee, Ill.) contained 0.32 mg of iron per 1 mg of the protein. Imferon (iron dextran complex; Lakeside Laboratories, Inc., Milwaukee, Wisc.) contained 50 mg of iron per 1 ml of the solution. Blutal (iron chondroitin sulfate colloid), containing 4 mg of iron per 1 ml of solution, was produced by Dainippon Pharmaceutical Co., Osaka, Japan, and was kindly provided for this work by A. Wake.

Antimycobacterial assay and its alleviation. Tests to determine the ability of various iron-carrying materials to neutralize serum- or Tween 80-induced tuberculostasis were performed by the agar plate diffusion test (12). In this test, 9 ml of iron-poor Dubos agar medium was mixed with 3 ml of saline, bovine serum, or dilutions of Tween 80. The mixtures were incubated at 56 C for 30 min. After incubation, 2 ml of the mixture was transferred to a plastic petri dish (no. 3002, 60 by 15 mm; Falcon Plastics) and allowed to solidify. A well, 10 mm in diameter, was made by placing a siliconized glass cylinder in the center of the solidified base layer and then pouring the remaining 10 ml of medium into the plate. After solidification of the medium, the plates were incubated overnight at 37 C to permit evaporation of moisture on the surface of the solidified agar medium.

Plates were inoculated with 0.25 ml of a Klett 1 suspension of BCG, H37Ra, or H37Rv bacilli. After the water of the inoculum was absorbed by the medium, the glass cylinders were removed and the wells were charged with 0.4 ml of the test materials. The plates were reincubated at 37 C in a 75%-humidity chamber. After a 21-day incubation period, the plates were examined for bacillary growth or growth inhibition around charged wells. Results were recorded by photographing plates placed on a template which had a scale indicating 5-mm intervals.

Spent media. Spent media were prepared by growing BCG, H37Ra and H37Rv bacilli in iron-poor Dubos medium which contained various concentrations of Triton WR-1339 (Rohm and Haas Co., Philadelphia). Media were inoculated with 0.1 ml of Klett 80 bacillary suspensions and incubated for 14 days at 37 C. After the incubation, the bacillary cultures were adjusted to equal densities, the bacilli were removed by filtration, and spent media were tested for the neutralizing quality against serum tuberculostasis. In such test, the tuberculostasis-neutralizing quality of spent media would indicate the quantity of M released by the same number of BCG, H37Ra, and H37Rv cells.

Ethanol-Tween and lecithin extracts of tubercle bacilli. The alleviation of serum tuberculostasis by ethanol-Tween and lecithin extracts of BCG, H37Ra, and H37Rv bacilli was determined by the agar plate diffusion test. Extracts were prepared by subjecting 1 g of moist weight bacilli, grown on the surface of iron-poor Dubos agar medium, to washing with 10 ml of 70% ethyl alcohol containing 0.05% Tween 80 or 3 ml of saline-solubilized bovine lecithin (90% pure, Nutritional Biochemicals Corp.). The bacillary suspensions were mixed with a magnetic stirrer during a 24-h extraction period. After the extraction, bacilli were removed from extracts by centrifugation, the alcohol was evaporated, and the extracts were adjusted to 3-ml volumes with saline.

Amount of lipid on bacillary cells. BCG, H37Ra, and H37Rv bacilli were harvested from the surface of iron-poor Dubos agar medium, and the cells were washed with saline and dried overnight at 75 C. Total lipid present in 2 g of dried bacillary mass was determined by the method of Anderson (1). Each bacillary mass was successively extracted with 20 ml of the following solutions: diethyl ether-ethyl alcohol (1:1, vol/vol), chloroform, and chloroform-methyl alcohol (2:1, vol/vol). The extracts were dried, and the amount of lipoidal material was determined by weighing.

RESULTS

Previous findings in our laboratory have shown that the fate of tubercle bacilli in mammalian serum was determined by the interaction between Tr and M (15). M promotes the bacillary multiplication by removing iron from Tr and supplying the metal for bacillary utilization. Since tubercle bacilli are facultative intracellular parasites and iron in cells is stored in F molecules, it became necessary to determine the mechanism which enabled tubercle bacilli to obtain growth-essential iron in phagocytic cells of animal body.

As the first step in this study, we determined whether iron of F could alleviate iron-determined tuberculostasis of bovine serum for BCG bacilli. Solutions of ferric ammonium citrate, F, Imferon, and Blutal were administered to wells made in tuberculostatic medium composed of bovine serum diluted 1:4 in iron-poor Dubos agar medium. The addition of iron-containing Imferon and Blutal neutralized the serum tuberculostasis as effectively as the addition of

ferric ammonium citrate (Fig. 1). This finding indicates that iron of Blutal and Imferon is as easily available for bacillary utilization as ionic iron of ferric ammonium citrate. Although large quantities of F-stored iron were used, the degree of neutralization of serum tuberculostasis was insignificant; to obtain Blutal- or Imferon-equivalent neutralization of tuberculostasis with F, about 50 times more F-iron was required than the amount present in Blutal or Imferon. The possibility of the presence of tuberculostasis neutralizing ionic iron in Blutal and Imferon was eliminated by the dialysis experiment; dialyzed iron-containing compounds were as effective in the neutralization of serum tuberculostasis as they were before dialysis (Fig. 1).

The inability of F to alleviate serum tuberculostasis showed that tubercle bacilli surrounded by F, in which iron constituted 32% of its dry weight, were unable to obtain the growth-essential iron. In subsequent experiments, therefore, we tried to alleviate serum tuberculostasis in the agar plate diffusion test by charging the wells with F-M mixtures. A concentration of M, which would not alleviate tuberculostasis of bovine serum with 76% of unsaturated Tr, was mixed with various concentrations of F; the mixtures were incubated for 1 h at 37 C, and then were tested for alleviation of tuberculostasis. The tuberculostasis was neutralized around wells which received F-M mixtures but not around wells with F alone (Fig. 2). A small area of neutralization around the well charged with 0.5 mg of F might be due to slow dissociation of iron during the 3-week incubation period. The comparison between degrees of the serum neutralization induced with ferric ammonium citrate (12) and with F-M mixture indicates that M could make all iron in F molecule available for bacillary utilization. The limited effectiveness of the neutralization around wells with large quantities of F (0.5 mg) and small amount of M (1.0 μg/well) is due to the limitation of M rather than to unavailability of iron.

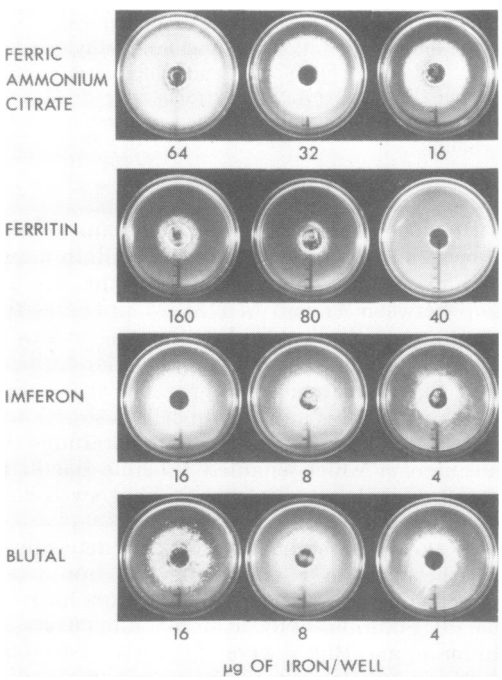

FIG. 1. *Neutralization of serum tuberculostasis around wells charged with various concentrations of iron-containing materials (expressed in terms of their iron content).*

Since M can remove iron from F, a possibility existed that the presence of an iron carrier, analogous to M, in phagocytic cells could satisfy the needs for the metal of not only the cells but also of tubercle bacilli. This possibility was investigated by a 1-h-incubated mixture composed of F and macrophage lysates. The lysates were prepared by subjecting the peritoneal and alveolar macrophages to 10 successive freeze-thaw cycles (13). The F-macrophage lysate mixtures were tested for the neutralization of serum tuberculostasis by the agar plate diffusion test. Results showed that F with or without macrophage lysates did not appreciably neutralize serum tuberculostasis (Fig. 2). This study showed that macrophages possess no iron carrier which would supply intracellular tubercle bacilli with iron and, therefore, the parasites are dependent upon M which supplies them with the metal by removing it from F.

The crucial role of M in the survival of tubercle bacilli suggested that this bacillary product may determine the fate of virulent and

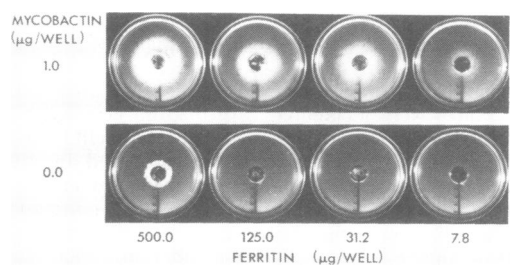

FIG. 2. *Neutralization of serum tuberculostasis around wells charged with various concentrations of ferritin and ferritin-mycobactin solutions. Wells charged with ferritin-macrophage lysate mixtures did not neutralize serum tuberculostasis, and results were identical to those obtained with ferritin alone.*

avirulent bacilli in hosts. It is possible that virulent bacilli survive in the host because either they possess more M or M is associated with virulent bacilli differently than with avirulent bacilli. It has been shown by Golden (Ph.D. thesis, Miami Univ., Oxford, Ohio, 1973) that the amount of M in H37Ra and H37Rv bacilli is the same. A study performed by Snow (19) showed that the yields of M vary considerably with bacillary strains; very frequently, Snow extracted more M from avirulent than from virulent tubercle bacilli. We concluded, therefore, that the survival of bacilli in the host is not determined by the amount of M present on bacillary cell.

In this study we investigated the association of M with BCG, H37Ra, and H37Rv bacilli in the presence of surfactants which were shown to remove M from bacillary cells possibly by facilitating the solubility of this hydrophobic material (12). Each strain of tubercle bacilli was inoculated onto the surface of iron-poor Dubos agar medium which contained different concentrations of Tween 80 (Table 1). Wells were charged with saline, 160 μg of iron, or 40 μg of M. The bacillary growth on plates in which wells were charged with saline indicated that H37Rv was the strain most resistant to the effect of Tween 80; although a concentration of 1.0% Tween was required to inhibit the growth of H37Rv bacilli, the growth of BCG and H37Ra bacilli was inhibited by one-third and one-fifth this concentration, respectively.

The inhibition of H37Ra and BCG multiplication at low concentrations of Tween 80 could be effectively neutralized by both iron and M (Table 1). However, at high concentrations of Tween 80, iron and M failed to overcome the effects of Tween 80. The exposure of bacilli to high Tween 80 concentrations was not lethal; when the bacilli were removed from the Tween-containing medium and plated on the Tween-void Dubos agar medium, they grew unhindered. The failure of Tween-exposed bacilli to grow around wells charged with high amounts of M or iron indicates that the surfactant not only removes M from bacillary cell but also it interferes with the absorption of M-iron complexes.

Twenty-five years ago, Dubos and Middlebrook showed that avirulent bacilli are inhibited by concentrations of Triton which permitted the growth of virulent bacilli (6). The results obtained with Tween 80 suggested that the loss of M from bacillary cells could account for the observations of Dubos and Middlebrook. The validity of this suggestion was tested by growing BCG, H37Ra, and H37Rv bacilli in iron-poor Dubos medium (without agar) in the presence of various concentrations of Triton WR-1339. Spent media were tested for their ability to neutralize the serum tuberculostasis in plates of agar plate diffusion test. Since spent media were adjusted to the same bacillary densities, the serum tuberculostasis-neutralizing effect of spent media would reflect the amount of M released by each strain of tubercle bacillus. With each higher concentration of Triton in the medium, wider zone of bacillary growth was present around spent medium-charged wells (Table 2). Both H37Ra and BCG spent media effectively neutralized serum tuberculostasis.

TABLE 1. *Tuberculostatic effect of Tween 80 on the growth of BCG, H37Ra, and H37Rv bacilli and the alleviation of tuberculostasis by iron or mycobactin (M)*

Tween 80 (%)[a]	Bacillary strain	Width (mm) of growth around wells containing[b]		
		Saline	Iron	M
1.0	BCG	0	0	0
	H37Ra	0	0	0
	H37Rv	0	0	0
0.5	BCG	0	0	0
	H37Ra	0	7	0
	H37Rv	22[c]	22	22
0.4	BCG	0	0	5
	H37Ra	0	22	17
	H37Rv	22	22	22
0.3	BCG	0	11	9
	H37Ra	0	22	20
	H37Rv	22	22	22
0.2	BCG	22	22	22
	H37Ra	0	22	22
	H37Rv	22	22	22
0.1	BCG	22	22	22
	H37Ra	22	22	22
	H37Rv	22	22	22
0.0	BCG	22	22	22
	H37Ra	22	22	22
	H37Rv	22	22	22

[a] Bacilli exposed to these Tween 80 concentrations for 24 h in Dubos liquid medium were not killed and grew when plated on suitable medium.

[b] Wells made in iron-poor Dubos agar medium containing various concentrations of Tween 80 were charged with saline, 160 μg of iron, or 40 μg of M.

[c] Bacillary growth covering the entire plate had a width of 22 mm. Such uninhibited growth of H37Rv strain was present on media containing less than 1% of Tween 80.

TABLE 2. *Growth of BCG bacilli on tuberculostatic serum-agar medium around wells which received Triton-containing spent media of BCG, H37Ra, and H37Rv bacilli*

Triton in media[a] (%)	Width (mm) of BCG growth around wells charged with spent medium of		
	BCG	H37Ra	H37Rv
0.50	15	17	9
0.10	11	13	2
0.05	8	10	0
0.01	5	7	0
0.00	3	5	0

[a] Unused media containing similar concentrations of Triton failed to neutralize serum tuberculostasis.

TABLE 3. *Growth of BCG bacilli on tuberculostatic serum-agar medium around wells charged with lecithin and alcohol-Tween extracts of BCG, H37Ra, and H37Rv bacilli*

Extraction fluid	Width (mm) of BCG growth around wells charged with extracts of		
	BCG	H37Ra	H37Rv
Lecithin:			
5.00%	12	14	7
0.50%	10	11	6
0.05%	7	9	4
Ethanol-Tween	16	22	20
Saline	3	6	0

At each Triton concentration, the tuberculostasis-neutralizing activity of H37Ra spent medium was a little stronger than the BCG spent medium. In contrast to BCG and H37Ra spent media, the H37Rv spent media exerted little of tuberculostasis-neutralizing activity. It was surprising to find that, even in the absence of Triton, BCG and H37Ra spent media possessed some tuberculostasis-alleviating effect. The results show that virulent H37Rv bacilli release much less M in the presence of Triton than avirulent H37Ra or attenuated BCG bacilli.

The findings with surface-active agents may become pertinent to the survival of the tubercle bacillus in the host only if surfactants present in host tissues or fluids are capable of acting in a manner similar to that of Tween 80 and Triton. For this reason, an attempt was made to determine whether lecithin could remove M from bacillary cells. Avirulent, attenuated, and virulent strains of tubercle bacilli were extracted during a 24-h period with various concentrations of lecithin and ethanol-Tween solutions. The amounts of M in each extract were determined by testing the extracts for the neutralization of serum tuberculostasis in the agar plate diffusion test. We found that lecithin extracts of BCG and H37Ra cells neutralized similar areas on tuberculostatic medium and that therefore these extracts possessed similar content of M (Table 3). Extracts of H37Rv bacilli possessed much less tuberculostasis-neutralizing activity than extracts of BCG and H37Ra bacilli; the two latter strains released some M even when extracted with saline. Ethanol-Tween extraction was most efficient; the extracts of the three strains neutralized serum tuberculostasis with equal efficiency. These findings show that a naturally occurring surface-active agent lecithin can act in a manner similar to Tween 80 or Triton. The extraction with lecithin removed much more M from attenuated and avirulent bacilli than from virulent organisms. This finding becomes of considerable significance when one remembers that H37Ra and H37Rv bacilli possess equal amounts of M.

Experiments with Tween 80, Triton WR-1339, and lecithin showed that virulent bacilli lose much less M in the presence of these surfactants than do avirulent or attenuated bacilli. Since M is an extremely hydrophobic molecule, an important factor in determining the firmness of association between bacilli and M might be the quantity of lipid possessed by virulent and avirulent cells. The determination of lipid by the method of Anderson (1) showed that BCG strain possessed 260 mg, H37Ra strain possessed 130 mg, and H37Rv strain possessed 530 mg of lipid per 1 g of bacillary mass. Thus, the lipid content of virulent cells was four times as great as that of avirulent cells and two times as great as that of attenuated cells. This finding suggests that the high lipid content of virulent cells determines the high affinity of hydrophobic M to these bacteria.

DISCUSSION

The growth of tubercle bacilli in a host is determined by the ability of the parasites to utilize fluids and tissues of the host as a source of essential nutrients. Studies in our laboratory showed that mammalian sera do not support the growth of tubercle bacilli because they fail to provide the parasites with growth-essential iron (10, 12). However, when tuberculostatic sera were supplemented with iron or M, they supported bacillary multiplication. Iron in serum is bound to Tr and can be utilized by bacilli only in the presence of M (15).

Results presented in the first part of this report show that the role of F in the intracellular

multiplication of tubercle bacilli is quite analogous to the role of Tr in mammalian serum-suspended tubercle bacilli; both Tr and F retain iron which is necessary for bacillary utilization. M can remove iron from Tr and F in spite of the fact that the metal is bound firmly to Tr and deposited as quite insoluble ferric hydroxide-phosphate complex within a protein shell of F (7). In contrast to iron present in Tr and F, iron bound to dextran (Imferon) or to chondroitin sulfate (Blutal) is available for bacillary utilization. It is possible that iron in Imferon and Blutal is located in such a way that M, present in the cell wall, can establish a contact with the metal and remove it by chelation. These results suggest that Blutal and Imferon should replace various iron salts used in the past to increase the susceptibility of animals to various infectious diseases. Not only is iron in both substances quite available for bacillary utilization, but it is also less toxic to the treated animals.

Attempts to reveal an iron carrier in serum or in macrophage lysates with an ability to supply iron of Tr or F to tubercle bacilli were unsuccessful. This study does not explain, therefore, how virulent bacilli obtain iron in infected macrophages. The possibility exists that acidic pH values in infected cells favor the dissociation of iron from F in sufficient amounts for the initiation of bacillary growth; the iron starvation in the cells stimulates increased production of M (12), which provides bacilli with enough metal for unhindered multiplication.

The second part of this report examines M not only as the essential factor for bacillary growth but also as a factor determining virulence of tubercle bacilli. Repeated ethanol-Tween 80 extractions of avirulent and virulent bacilli showed equal amounts of M, irrespective of bacillary virulence (C. A. Golden, Ph.D. thesis, Miami Univ., Oxford, Ohio, 1973). This lack of correlation between quantity of M and degree of virulence prompted the study pertaining to the firmness of association between M and virulent and avirulent tubercle bacilli. Experiments with Tween 80, Triton, and lecithin showed that BCG and H37Ra bacilli lose M at much lower concentrations of the surfactants than H37Rv bacilli. Since H37Ra and H37Rv bacilli possess equal amounts of M, we concluded that M is more firmly bound to virulent than to avirulent cells.

It is interesting that the additions of M to inhibited bacilli at high concentrations of Tween 80 is ineffective, although M or iron additions to bacilli at low concentrations promoted bacillary multiplication (Table 1). This observation indicates that, at high concentrations of Tween 80, either M cannot remove iron from Tr, or M-iron complexes cannot be absorbed by tubercle bacilli. The nature of Tween 80 would suggest that it is the latter possibility. Since nonionic surface-active agents, both synthetic and natural, contain fat-soluble and water-soluble groups in the same molecule, these materials have the potential to influence the relationship between M and lipid of bacterial cell wall. The presented results indicate that these surfactants not only facilitate dispersion of M from bacterial wall, but that they also interfere with the adsorption of M-iron complex by reacting with cell wall lipid. The four-times-larger content of lipid in virulent than in avirulent bacilli prevents rapid loss of M from disease-producing bacteria; in avirulent cells, M is lost rapidly and is replaced by surfactant which interferes with the adsorption of M-iron complexes.

The effects of various surface-active agents on tubercle bacilli have been investigated by several investigators. It has been observed that Tween 80 promotes dispersed growth of tubercle bacilli, and this action was attributed either to an alteration of the surface charge or structure of the organism (5). Although a change in cell structure at low Tween 80 concentrations has not been demonstrated (18), Bloch and Noll (3) have shown that 2.0% of Tween 80 modified surface structures of tubercle bacilli and reduced their virulence. They demonstrated that, under these conditions, loss of virulence was associated with the removal of a lipid component "cord factor" of the surface material. Our study shows that Tween treatment of bacilli removes M from their surfaces. It is possible that the reduction of virulence in the Tween-treated bacilli was due to the loss of growth-essential M rather than to the functionally undefined cord factor.

The study of antituberculous effects of synthetic nonionic surface-active compounds, belonging to Triton family, has been stimulated with the observations which showed that Triton WR-1339 suppresses the development of experimental tuberculosis in mice (4). This antituberculous activity of Triton WR-1339 was attributed to a modification of bacillary surface, which facilitated the digestion of the parasites by intracellular enzymes (9). A recent study by Hart and Payne (8) showed that Triton WR-1339 inhibits the activity of lipases in lysosomes of macrophages where the confrontation between host and parasites takes place. Although these authors suggested that the beneficial effect of surfactants was due to protection of host rather than to damage of bacterial cell,

they indicated that the mechanism which would explain satisfactorily the antituberculous activity of certain surfactants is still obscure. We found that Triton WR-1339 removes M from bacillary cell and, therefore, its antimycobacterial activity could be attributed to the inactivation of the iron-providing mechanism. This effect as well as the interference of Triton WR-1339 with M-iron transport into the bacillary cell could constitute an effective antituberculous mechanism in treated animals. In view of this discussion, the resistance of virulent bacilli to M-removing activity of Tween 80, Triton WR-1339, and lecithin can be an indicator of their ability to survive in the host and, therefore, the measure of their virulence.

The most effective defense mechanism of a host against "iron-dependent" parasites would be the production of antibodies to bacterial chelators which provide the parasites with growth-essential iron. It has been demonstrated that vaccinated animals possess low concentrations of an "incomplete" antibody whose levels correlated with the degrees of resistance to challenge with virulent bacilli (17). These antibodies are formed against haptenic lipoidal material present in the methanol extract of mycobacterial cells. Although the methanol-soluble material was characterized as a phosphoglycolipid, it is possible that the preparations contained the methanol-soluble M whose iron-supplying activities were stopped by the specific antibodies. Attempts will be made, therefore, to stimulate production of antibodies to hapten-like M with techniques which will endow the molecule with potent immunogenic activity.

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