THE EFFECT OF SPHINGOMYELIN ON THE GROWTH OF TUBERCLE BACILLI

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The enhancing effect of the synthetic wetting agents Tween 40, Tween 60, and Tween 80 on the growth of tubercle bacilli is due in part to the fact that these water-dispersible esters constitute for the bacteria a non-toxic source of palmitic, stearic, or oleic acid. Indeed, enhancement of growth can also be observed with the sodium soaps of these same fatty acids, as well as of linoleic, linolenic, and arachidonic acids, provided that the bacilli are protected against the toxic action of these soaps, for example by the addition of serum albumin to the medium (6).

In addition to long chain fatty acids, there exist in tissues other lipids which enhance the growth of tubercle bacilli (1, 2, 5, 10). Further study of this problem has led to the recognition that, in this respect, sphingomyelin is more active than any of the other naturally occurring phospholipids and cerebrosides tested in our laboratory. It will be shown in the present paper that the addition to simple culture media of sphingomyelin prepared from various tissues markedly increases the density of growth within a short period of incubation and also facilitates initiation of growth from small inocula, even in the absence of serum albumin or other protein.

EXPERIMENTAL

All experiments described in the present report were carried out with a virulent culture of the strain of human tubercle bacillus H37Rv recently recovered from infected mouse lung and maintained in the Tween-albumin medium described elsewhere (8). The 10 day old culture used for inoculation consisted of finely dispersed bacilli and contained approximately 0.35 mg. bacillary cells (dry weight) per cc. of medium.

The basal medium used in the experiments had the following composition:

KH_2PO_4	1.0	gm.	heat in 100 cc. distilled water to dissolve
$Na_2HPO_4 \cdot 12\ H_2O$	6.3	"	
Asparagine	1.0	"	
Add:			
Distilled water	850 cc.		
Enzymatic digest of casein	0.5	gm.	(10 cc. of a 5 per cent solution in distilled water)
$ZnSO_4$	0.001	"	(1 cc. of a 0.01 per cent solution in distilled water)
Ferric ammonium citrate	0.005	"	
$MgSO_4 \cdot 7\ H_2O$	0.01	"	(1 cc. of a 1 per cent solution in distilled water)

$CaCl_2$	0.0005 gm.	(1 cc. of a 0.05 per cent solution in distilled water)
$CuSO_4$	0.0001 "	1 cc. of a 0.01 per cent solution in distilled water)

Adjust pH to 6.5

It was distributed in 3 cc. amounts in pyrex glass tubes 25 mm. in diameter. For reasons given in an earlier publication, aluminum caps were used instead of cotton plugs (7). The other ingredients were added, as indicated in the text, either before autoclaving or aseptically as sterile solutions.

Serum albumin was the fraction V of bovine plasma. It was dissolved in a concentration of 5 per cent distilled water. The solution was sterilized by filtration through a porcelain candle.[1]

The non-ionic wetting agent Triton A20[2] was added to the medium in a number of tests. This wetting agent is a heat-stable arylalkyl polyether of phenol which disperses the cultures of tubercle bacilli without increasing the yield of growth. Its properties will be further discussed in a subsequent publication.

Most of the preparations of sphingomyelin, cerebroside, lignoceric amide, lignoceric acid, and sphingosine used in the present study were obtained from the chemical collection of the late Dr. P. A. Levene maintained at the Rockefeller Institute. The chemical structure and properties of these substances as well as the methods involved in their preparation have been described in references 9 and 11. A sample of pure lung sphingomyelin was generously supplied by Dr. S. J. Thannhauser of Joseph H. Pratt Diagnostic Hospital, Boston; this material had been freed of contaminating dipalmityl glyceride by the technique described in reference 12.

All preparations of lipids were dispersed in distilled water starting from chloroform solutions whenever necessary. Sphingomyelins and cerebrosides were sterilized by three consecutive heatings at 80°C., although more recent tests indicate that their effect on the growth of tubercle bacilli is not appreciably modified by autoclaving in the basal medium.

All lipids imparted a certain degree of opalescence to the medium. It was observed that complete dispersion of sphingomyelin, resulting in a clear medium, could be obtained by adding to the medium the wetting agent Triton A20 (see above) in a final concentration of 0.01 to 0.02 per cent.

Effect of Sphingomyelin and Cerebroside Preparations on the Growth of Tubercle Bacilli.—The yield of bacilli within a given period of incubation and the ability of the medium to allow proliferation of very small inocula have been used as criteria in the following experiment to compare the growth-promoting properties of sphingomyelins and cerebrosides and of serum albumin.

The preparations of sphingomyelin and cerebroside to be tested were added in 0.3 cc. amounts of emulsions of various concentrations to 3 cc. of the basal medium. Albumin (0.3 cc. of 5 per cent solution), or water, was used for the control media. Four sets of each medium were prepared; to two had been added 0.02 per cent Triton A20 prior to autoclaving. The tubes were inoculated with 0.003 or 0.000003 cc. of a 10 day old culture of H37Rv in Tween albumin medium, diluted in 0.3 cc. distilled water. These inocula corresponded approximately to 3×10^{-4} and 3×10^{-7} mg. of dry bacilli per cc. of medium. Final readings of

[1] Bovine albumin (serum fraction V) was obtained in a desiccated form from Armour Laboratories, Chicago.

[2] Triton A20 was generously supplied by Rohm and Haas Company, Philadelphia.

macroscopic evidence of growth, confirmed in some cases by microscopic examination, were made after 10 days' incubation at 37°C.

TABLE I

Comparative Effects of Sphingomyelins, Cerebrosides, and Serum Albumin on the Growth of Tubercle Bacilli

Test substances added to the medium			Inoculum H37Rv (mg. dry bacilli per cc. medium)			
			3×10^{-4}		3×10^{-7}	
source*	Final concentration		Basal medium	Basal medium + 0.02 per cent Triton A20	Basal medium	Basal medium + 0.02 per cent Triton A20
	per cent					
Kidney sphingomyelin	P.A.L. (1126)	0.05	5‡	7‡	2‡	3‡
		0.01	3	5	1	2
Liver "	" (1127)	0.05	6	8	2	2
		0.01	4	6	2	3
Brain "	" (1131)	0.05	5	7	2	3
		0.01	3	5	1	3
Lung "	Thannhauser	0.05	5	7	2	3
		0.01	3	5	2	3
Cerebron	P.A.L. (928)	0.05	2	2	0	0
		0.01	3	5	2	3
Cerebron	" (932)	0.05	1	0	0	0
		0.01	2	2	0	0
Phrenosin	" (1115)	0.05	2	1	0	0
		0.01	2	1	0	0
Serum albumin		0.5	7	8	3	4
H_2O			2	2	0	0

* The initials P.A.L. indicate that the material was obtained from the chemical collection of the late Dr. P. A. Levene maintained at the Rockefeller Institute. The number in parentheses refers to the classification number in this collection. The sample of lung sphingomyelin was received from Dr. S. J. Thannhauser of Boston.

‡ The amount of growth is indicated in terms of an arbitrary ascending scale (from 0 to 8) based on gross macroscopic examination. The figure 8 corresponds to approximately 0.4 mg. dry bacilli per cc. of medium.

The results presented in Table I show that, after 10 days' incubation, growth in the absence of either serum or lipid could be detected only in the tubes having received the larger inoculum. At that time, growth was abundant in all the

albumin media irrespective of the size of the inoculum. The cerebroside preparations did not improve, and in fact decreased, the ability of the medium to support the growth of the small inocula. All preparations of sphingomyelin, on the contrary, were almost as effective as serum albumin in enhancing the yield of bacilli and in allowing the proliferation of the small inocula. Addition to the medium of the wetting agent Triton A20 improved the performance of sphingomyelin on several accounts: (*a*) it dispersed the phospholipid, thereby rendering the medium more limpid; (*b*) it increased the bacillary yield; (*c*) it

TABLE II

Comparative Protective Effects of Sphingomyelin and Albumin against the Toxicity of Fatty Acids

Final concentration in basal medium of			Growth 10 days after inoculation with 3×10^{-4} mg. H37Rv per cc. medium
Fatty acid	Albumin	Sphingomyelin	
per cent	*per cent*	*per cent*	
Oleic acid 0.03	0	0	0*
" " "	0	0.1	7
" " "	0.5	0	8
" " 0.01	0	0	0
" " "	0	0.1	7
" " "	0.5	0	7
Lauric acid 0.01	0	0	0
" " "	0	0.1	5
" " "	0.5	0	6
Capric acid 0.01	0	0	0
" " "	0	0.1	5
" " "	0.5	0	6
H_2O	0	0	3
"	0	0.1	5
"	0.5	0	6

* Symbols same as in Table I.

caused the growth to be fairly well dispersed (diffuse growth) instead of being granular as was the case in media containing either albumin or lipids without the wetting agent.

The Neutralizing Effect of Sphingomyelin on the Toxicity of Long Chain Fatty Acids.—As shown in earlier publications, serum albumin promotes the proliferation of small inocula of tubercle bacilli not necessarily by supplying nutritive factors, but rather by protecting the organisms against the toxic effect of various injurious agents (3, 4, 7). The following experiment reveals that sphingomyelin can exert a similar protective effect against the toxicity of long chain fatty acids.

The sodium soaps of caproic, lauric, or oleic acid were added to the basal medium in the concentrations indicated in Table II. Different samples of sphingomyelin were added in

amounts of 0.3 cc. of 1 per cent emulsion per 3 cc. of medium, in comparison with 0.3 cc. of 5 per cent albumin or 0.3 cc. of water. All tubes were inoculated with 0.003 cc. of H37Rv culture in Tween-albumin medium diluted in 0.3 cc. distilled water (corresponding approximately to a final inoculum of 3×10^{-4} mg. dry bacilli per cc. of medium). Macroscopic evidence of growth was read after 10 days' incubation at 37°C. The results are recorded in Table II (the data refer to only one of the preparations of sphingomyelin tested: P.A.L. 1127).

The results presented in Table II confirm earlier findings that the inhibitory effect of long chain fatty acids on the growth of tubercle bacilli can be neutralized by addition of serum albumin to the medium and establish that sphingomyelin exhibits a similar protective effect. It is of interest in this respect that addition of this phospholipid to emulsions of sodium soaps increases appreciably the apparent water solubility of the latter, suggesting the formation of a complex such as has been recognized to occur between fatty acids and albumin.

The Effect of Lignoceric Acid on the Growth of Tubercle Bacilli.—Sphingomyelin is a diaminophospholipid in which a C24 hydroxy acid (lignoceric acid) is combined in amide linkage with the base sphingosine, phosphocholine being esterified on the latter. We have not yet studied the effect of phosphocholine on bacterial growth. All samples of sphingosine tested so far have failed to enhance and indeed have caused a marked inhibition of growth; the nature of this inhibition will be considered in a subsequent publication. On the contrary, both lignoceric acid and its amide have been found to exert a beneficial effect on growth as shown in the following experiment.

Solutions of lignoceric acid and lignoceric amide in chloroform were added in graded amounts to the basal medium containing 0.02 per cent of Triton A20. Constant agitation while heating to eliminate the chloroform yielded a fairly stable emulsion of the lipids in the medium, despite their very low water solubility. The media were distributed in 4 cc. amounts in test tubes (25 mm. diameter) and autoclaved. Serum albumin was then added in final concentrations of 0.5 per cent to a duplicate set. The inoculum was 0.04 cc. or 0.004 cc. of a 10 day old culture of H37Rv diluted in 0.4 cc. distilled water (this corresponded approximately to inocula of 3×10^{-3} or 3×10^{-5} mg. dry bacilli per cc. of final medium). Macroscopic evidence of growth was recorded after 10 days' incubation at 37°C.

The results presented in Table III establish that, like sphingomyelin and oleic acid, lignoceric acid and its amide can enhance the growth of tubercle bacilli. The latter substances allow growth of small inocula and are therefore less toxic than oleic acid. This may be due to their very low solubility in water and, in the case of lignoceric amide, to the fact that the carboxyl group is masked.

On the basis of the information summarized in Tables II and III, it appears therefore that sphingomyelin exerts a favorable effect on growth by a dual mechanism. Like serum albumin, on the one hand, it protects the bacilli against certain toxic effects, in particular those of long chain fatty acids. On the other hand it constitutes a water-dispersible source of lignoceric acid which is available for metabolic utilization. In addition to their bearing on the

metabolism of tubercle bacilli, these facts may be of significance in the analysis of the factors which condition the proliferation of tubercle bacilli *in vivo*. Although sphingomyelin was first recognized in nervous tissue, it appears to be a constituent of many if not of all types of cells; and it is probable that its release from these cells at the site of infection and in caseous material would not be without effect on the growth of the bacilli.

TABLE III

Comparative Effects of Sphingomyelin, Lignoceric Acid and Amide, Sphingosine and Oleic Acid on the Growth of Tubercle Bacilli

Test substances added to the medium			Inoculum H37Rv (mg. dry bacilli per cc. of medium)			
			3×10^{-3}		3×10^{-4}	
	Source*	Final concentration	Basal medium	Basal medium + 0.5 per cent albumin	Basal medium	Basal medium + 0.5 per cent albumin
		per cent				
Sphingomyelin	P.A.L. (1127)	0.02	8*	8*	3*	8*
Lignoceric acid	" (1076)	0.01	7	8	2	8
" "	" (1077)	"	7	8	2	8
" amide	" (1074)	0.012	7	8	3	8
Sphingosine	" (1140)	0.01	0	4	0	0
Oleic acid		"	0	8	0	6
" "		0.003	0	8	0	6
H_2O			4	6	0	4

* Symbols same as in Table I.

SUMMARY

All preparations of sphingomyelin tested, whatever the tissues from which they originated, were found to enhance the growth of tubercle bacilli *in vitro*. Cerebrosides were inactive in this respect.

Sphingomyelin promotes growth through two independent mechanisms:

(*a*) It neutralizes the toxicity of long chain fatty acids probably by forming with them inert complexes. This protective effect facilitates initiation of growth from small inocula.

(*b*) It supplies to the bacteria lignoceric acid (or its amide) which is utilized for growth. The base sphingosine, another component of sphingomyelin, does not favor and probably inhibits proliferation of tubercle bacilli.

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