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ROLE OF LIPID CONTENT AND HYDROGEN PEROXIDE SUSCEPTIBILITY IN DETERMINING THE GUINEA-PIG VIRULENCE OF *MYCOBACTERIUM TUBERCULOSIS*

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Summary.—Among isoniazid-sensitive strains of *Mycobacterium tuberculosis*, strong associations were found in 56 strains of phage type A and I from India, Burma and East Africa between attenuation in the guinea-pig, a low content of strongly acidic (SAL) and sulphatide (SL) lipids, the presence of the attenuation indicator (AI) lipid and phage type I, suggesting that lipid content might mediate attenuation. However, 22 strains of phage type B and I from Iran and Britain also had low contents of SAL and SL but were highly virulent. Although the finding of a strong association in all 78 strains between attenuation and H_2O_2 susceptibility *in vitro* supports other evidence that attenuation is often due to increased susceptibility to H_2O_2 secreted by macrophages, an alternative mediator of virulence probably exists since 8 of the strains were attenuated and also resistant to H_2O_2. Identification of South Indian attenuated strains for epidemiological purposes by *in vitro* tests would be best achieved by the presence of AI, H_2O_2 susceptibility and, if the strains originated in or near India, by phage type I.

AN IMPORTANT CATEGORY of strains of *M. tuberculosis* that are attenuated in the guinea-pig is one in which loss of virulence is associated with increased susceptibility to the bactericidal activity of H_2O_2 *in vitro* (Mitchison, 1964). Some isoniazid-sensitive strains in this category are commonly found in India (Bhatia *et al.*, 1961; Mitchison, Selkon and Lloyd, 1963; Nair *et al.*, 1964) and less frequently in nearby countries such as Burma and East Africa (Mitchison, 1970) and have often been called "South Indian" strains. In these areas, strains that are highly virulent and H_2O_2-resistant are also present. Other attenuated strains, of world-wide distribution, are isoniazid-resistant and their susceptibility to H_2O_2 is accompanied by loss of catalase activity (Bonicke, 1954; Cohn *et al.*, 1954; Knox, Meadow and Worssam, 1956; Kreis and Le Joubioux, 1957). There is now strong evidence that H_2O_2 generated by macrophages is responsible for killing tubercle bacilli that they have phagocytosed, so that attenuation in the guinea-pig can be assumed to be the direct result of increased bacterial susceptibility to H_2O_2 (Coleman and Middlebrook, 1956; Mitchison *et al.*, 1963; Jackett *et al.*, 1981; Walker and Lowrie, 1981). Nevertheless, for reasons including the existence of strains such as BCG and H37Ra, which are attenuated but retain their resistance to H_2O_2 (Jackett, Aber and Lowrie, 1978), and the purely bacteriostatic action of immunity in chronic murine tuberculosis (Rees and Hart, 1961), attenuation of tubercle bacilli is likely to be mediated not only by increased susceptibility to H_2O_2 but by other additional characteristics (Hart, 1982). One such characteristic could be their lipid content. Goren *et al.* (1976) have suggested that lipids, particularly sulphatides, might

contribute to pathogenicity by inhibiting phagosome-lysosome fusion and by potentiating the toxic effects of cord factor on mitochondria (Kato and Goren, 1974). This possibility was strengthened by the finding, in Indian strains, that attenuation in the guinea-pig was associated with a low concentration of strongly acidic (SAL) and sulphate (SL) lipids and, even more strongly, with the presence of lipid characterized as the *O*-methyl ether of the aglycone moiety of mycosides A, B and G and named the "attenuation indicator" (AI) lipid (Goren, Brokl and Schaeffer, 1974*a*, *b*).

Bacteriophage typing of *M. tuberculosis* divides strains into 3 main types, A, B and I (intermediate). Most Indian strains are types A or I, while in Britain types A and B are frequent (Bates and Mitchison, 1969). The first part of the study, previously reported in part (Grange *et al.*, 1978), was done almost entirely on strains from India and surrounding countries. Attenuation, a low content of SAL and SL and, in particular, the presence of AI were found in phage type I strains, but rarely in those of phage type A. However, the same set of associations did not occur in the small sample of 4 type B Indian strains examined. Should it be usual for type B strains to have a low SAL and SL content and yet to be fully virulent in the guinea-pig, then it would seem unlikely that the similar low lipid content of phage type I strains was the direct cause of their attenuation. In an attempt to settle this question, a group of type B strains and a control group of type I strains whose virulence in the guinea-pig had been studied (Mitchison, 1970) were subjected to lipid analysis and to other *in vitro* tests found to be associated with virulence. These strains were obtained from patients in Britain and in Tehran, Iran, since phage type B is common in both countries. The results are reported here.

MATERIALS AND METHODS

Bacteria.—Strains of *M. tuberculosis* originating from patients in Madras, India, Rangoon, Burma or East Africa have been termed "Indian-type" since a proportion from each area have been found to be attenuated in the guinea-pig and to have other associated characters (Mitchison, 1970). Strains from Tehran, Iran and from Britain have been termed "Western-type", since they were always of high virulence. Methods for bacteriophage typing of strains of *M. tuberculosis* have been described previously (Grange, Collins and McSwiggan, 1976).

Studies.—Study 1, already reported on (Grange *et al.*, 1978) but with a different analysis of the data, was carried out on 56 isoniazid-sensitive Indian-type strains of *M. tuberculosis*, of which 21 were of phage type A, 31 of type I and 4 of type B. Study 2 was done later on 24 Western-type strains. Of these 19 were of phage type B, 10 from Britain, 7 from Tehran and the laboratory strains H37Rv (one culture from London and another from Denver) and H37Ra. The remaining 5 strains were of phage type I, 4 coming from Tehran and 1 from Britain. In addition to the 24 Western-type strains, 6 Indian-type strains which had been previously tested in Study 1 were included as controls of the technical methods used. All were isoniazid-sensitive and obtained from the patients before treatment was started (Mitchison, 1970). All strains were distributed for bacteriological and biochemical testing as coded isolates whose identity was not disclosed before statistical analysis.

Bacteriological and biochemical investigations.—The methods used to determine the virulence of strains in the guinea-pig, their susceptibility to H_2O_2, their sensitivity to thiophen-2-carboxylic acid hydrazide (TCH) and their content of SAL, SL and AI have been described previously (Grange *et al.*, 1978).

The virulence of the strains for the guinea-pig was measured as the extent of lesions in the organs 6 weeks after i.m. injection of 1·0 mg (moist wt) of bacilli; it is expressed as the "root-index of virulence" (RIV), (Mitchison *et al.*, 1961) in which a value of less than 1·0 is indicative of attenuation. Susceptibility to H_2O_2 was determined by counting colony-forming units (cfu) before and after exposure of a culture in 7H9 Middlebrook Tween–albumin medium (Difco Laboratories, Detroit Mich., U.S.A.) to 0·02% H_2O_2 for 60 min and is expressed as the percentage of cfu surviving the exposure (Subbaiah, Mitchison and Selkon, 1960). TCH in serial 2-fold concentrations was incorporated in Lowenstein–Jensen medium. The bacterial content of SAL and SL is given in neutral red activity (NRA) units (Goren *et al.*, 1974*a*). The AI lipid was detected according to Goren *et al.* (1974*b*).

RESULTS

The results with the laboratory strains of *M. tuberculosis* H37Rv and H37Ra have

TABLE I.—*Results of duplicate lipid analyses on the same strain*

Strain	Origin	Neutral red activity [units/g bacteria]: Strongly acidic lipid, Study 1	Strongly acidic lipid, Study 2	Sulphatide lipid, Study 1	Sulphatide lipid, Study 2	Attenuation indicator lipid
6792	Burma	0·5	1·0	0·4	0·1	+*
79112	Madras	8·5	9·5	8·0	7·3	+
7219	Madras	1·0	0·8	<0·1	0·4	+
6840	Burma	1·1	0·8	1·1	0·6	+
7201	Madras	0·4	0·9	<0·1	0·5	+
6827	E. Africa	0·3	0·9	0·1	<0·5	+
H37Rv†	U.S.A.	7·9	10·2	6·4	10·3	0

* 0, absent; +, present. The same results were obtained in both studies.
† Results on coded duplicates set up in Study 2.

been omitted from analyses, except that they are included in Tables I and IV. The lipid analyses of the 6 strains tested in both Studies 1 and 2 show good agreement between the duplicate estimations of SAL and SL (Table I), and there was complete agreement between the studies in the detection of AI. The clear distinction between the presence and absence of AI is shown in the Figure. The spot due to AI was distinguished from a less intense spot in the same position but unrelated to AI by the occurrence of a rose colour developing early in charring at 125–130°, by preparative thin-layer chromatography and by infra-red spectroscopy. A second more polar spot was always associated with the AI spot; the similarity of its colour reaction during charring and its infra-red spectroscopy pattern suggested that it was a monoacylated rather than the usual diacylated version of AI. The reproducibility of the lipid analyses was further indicated by the correct identification of 2 strains with unusual lipid patterns (79112, H37Rv) whose identities were unknown during laboratory analysis.

After exclusion of strains H37Rv and H37Ra and the duplicate results on the 6 strains tested in both studies, there remained 78 strains tested for all characteristics other than lipid content and 59 strains with lipid analyses. The SAL and SL content of Indian-type and Western-type strains are shown in Table II. Among Indian-type strains, groups with low and high lipid content were best distinguished by a critical NRA value of 4 units/g bacteria; low SAL and SL values were associated with attenuation in the guinea-pig. However, in Western-type strains, low lipid concentrations and high virulence occurred together. This association, the main objective of the study, was found not only in phage type B strains but also, surprisingly, in 4 of the 5 type I strains.

TABLE II.—*Lipid content of strains according to origin and root-index of virulence (RIV) in the guinea-pig*

Lipid	Strains	RIV	Neutral red activity (NRA) (Units/g bacteria): <1	1—	2—	3—	4—	6—	9 or more	Total
Strongly acidic (SAL)	Indian-type	1 or more	2	0	0	0	4	1	5	12
		<1	19	3	0	2	0	1	0	25
	Western-type	1 or more	4	7	4	2	4	0	0	21
		<1	0	0	0	0	0	1	0	1
Sulphatide (SL)	Indian-type	1 or more	2	0	0	1	4	2	3	12
		<1	20	2	0	2	0	1	0	25
	Western-type	1 or more	15	4	0	1	1	0	0	21
		<1	0	1	0	0	0	0	0	1

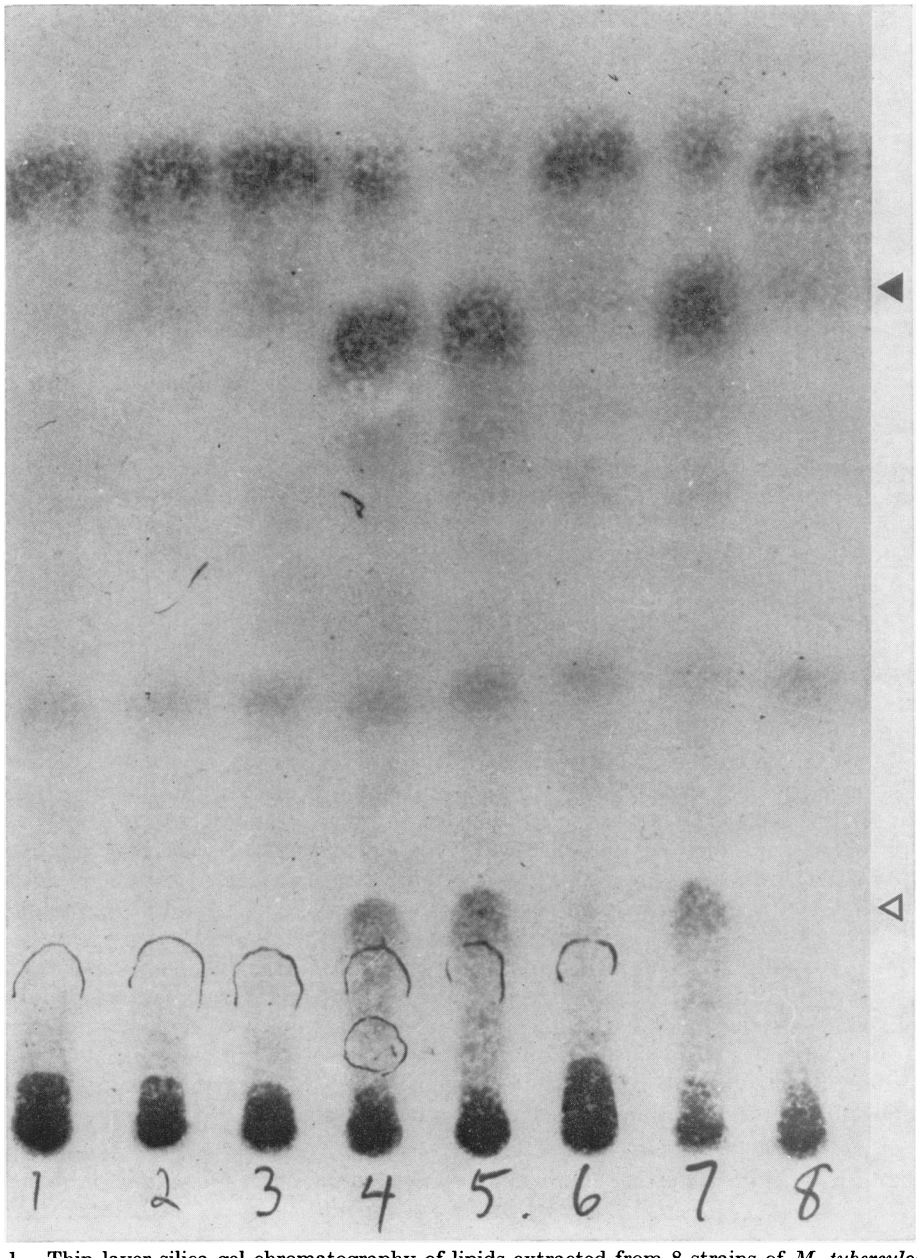

FIG. 1.—Thin layer silica gel chromatography of lipids extracted from 8 strains of *M. tuberculosis*. The attenuation indicator lipid (◀) and its monoacyl derivative (◁) are present in strains 4, 5 and 7. Crude extract lipids (100–200 g) were applied to 0·25 mm silica gel thin layer plates (SIL-G25, Brinkmann Instruments, Inc.), developed 12–15 cm in hexane: ether (9:1). dried briefly and sprayed with 60% H_2SO_4–0·2% (w/v) orcinol. On charring for 4—5 min at 130°, both AI lipids assumed a rose colour which ultimately blackened with prolonged heating.

TABLE III.—*Association between virulence in the guinea-pig, lipid content and other* in vitro *properties in Indian-type and Western-type strains of* M. tuberculosis

		Root-index of virulence (RIV) in the guinea-pig					
		Indian (RIV)		Western (RIV)		Total (RIV)	
		1·0 or more	<1·0	1·0 or more	<1·0	1·0 or more	<1·0
Susceptibility to H_2O_2 (%)	<10	0	26	2	1	2	27
(% survival)	10 or more	22	8	19	0	41	8
Attenuation indicator lipid	Present	0	22	1	0	1	22
	Absent	12	3	20	1	32	4
MIC of thiophen-2-carboxylic	<8	3	28	6	1	9	29
acid hydrazide (mg/l)	8 or more	19	6	15	0	34	6
Bacteriophage type	A	18	3	0	0	18	3
	I	2	29	4	1	6	30
	B	2	2	17	0	19	2
Strongly acidic lipid	<4	2	24	17	0	19	24
[NRA* units/g bacteria]	4 or more	10	1	4	1	14	2
Sulphatide lipid	<4	3	24	20	1	23	25
[NRA* units/g bacteria]	4 or more	9	1	1	0	10	1

* Neutral red activity

Characteristics of Indian-type and Western-type strains that might be associated with their virulence in the guinea-pig are set out in Table III. There are strong associations in both types of strain between attenuation and the proportion of organisms surviving exposure to H_2O_2 and also between attenuation and the presence of AI lipid. Thus, only 10 (13%) of the 78 strains had results discrepant from the association with H_2O_2 susceptibility and only 5 (8%) of the 59 strains examined had discrepant findings for the AI lipid. A weaker association is evident with sensitivity to TCH. Further, attenuated strains were more commonly phage type I than types A or B. As would be expected from consideration of the separate results with Indian and Western strains noted above, there was only a weak association in the combined results between attenuation and a low SAL and SL content.

In view of the strong evidence from other sources for a causative relationship between attenuation and susceptibility to H_2O_2, it is of interest to examine the complete results on 8 strains (6972-6903) which were attenuated (RIV <1·0) but resistant to H_2O_2 (>10%) (Table IV). Duplicate estimations of guinea-pig virulence and of H_2O_2 susceptibility are available for 3 of the strains, and these values agree with the initial estimates suggesting that technical error in the estimates is unlikely to have caused the discrepancies. Thus it seems that there is a small group of Indian-type strains which are attenuated despite being relatively resistant to H_2O_2. These have other characteristics associated with Indian-type strains, namely MICs of TCH of <8 mg/l, low SAL and SL content and AI present. There were also 2 Western-type strains (6213, 6045) which were virulent but susceptible to H_2O_2 with 8·8% and 2·1% surviving organisms respectively. It is uncertain whether these results are due to technical error or whether there is a small group of strains that retain virulence despite being susceptible to H_2O_2. The third group of strains considered in Table IV consists of 5 Western-type strains of phage type I (6045-6933) which differ from the previous description of type I Indian-type strains in that they are usually of high virulence (4 of 5 strains) resistant to H_2O_2 (3 of 5 strains) and lacking AI (4 of 5 strains). Finally, the results with strains H37Rv and H37Ra indicate that the attenuation that occurred in the latter

TABLE IV.—*Results of tests for virulence, hydrogen peroxide susceptibility, sensitivity to thiophen-2-carboxylic acid hydrazice (TCH) and lipid content for strains of* M. tuberculosis *giving results discrepant for virulence and peroxide susceptibility, for Western strains of phage type I and for strains H37Rv and H37Ra*

Strain No.	Origin	Phage type	Virulence (RIV)	H_2O_2 susceptibility (% survival)	MIC of TCH (mg/l)	Neutral red activity [units/g bacteria] strongly acidic lipid	Neutral red activity [units/g bacteria] sulphatide lipid	Attenuation indicator lipid
	Indian-type							
6972	BU*	I	0·99	16	1	—†	—	—
6896	BU	A	0·98	22	>64	—	—	—
7199	MA	I	0·78	18	8	0·3	<0·1	+
			0·82	48				
7075	MA	I	0·67	29	4	0·8	0·8	+
			0·95	28				
7026	BU	I	0·81	55	2	0·2	<0·1	+
6730	BU	I	0·89	66	2	<0·1	<0·1	+
7229	MA	I	0·69	66	4	1·0	1·0	+
			0·88	22				
6903	BU	B	0·94	87	2	0·7	0·7	+
	Western-type							
6213	BR	B	1·28	8·8	>64	4·0	0·5	0
6045	BR	I	1·22	2·1	>64	1·8	0·5	0
6721	TE	I	1·15	83	4	2·3	0·5	0
6912	TE	I	1·21	45	4	0·9	0·3	0
6626	TE	I	1·24	71	1	0·4	0·4	+
6933	TE	I	0·75	1·6	2	7·2	1·9	0
H37Rv	US	B	1·07	50	>32	7·9	6·4	0
H37Ra	US	B	0·52	100	—	0·3	0·3	0

* Burma; MA, Madras; BR, Britain; TE, Teheran; US, United States of America.
† —, not done; +, present; 0, absent.

after repeated laboratory subcultivation resulted in loss of SAL and SL but was not associated with the appearance of AI or with susceptibility to H_2O_2.

DISCUSSION

In the previous discussion of the results of the first part of the study (Grange *et al.*, 1978), 2 explanations were proposed for associations between phage type and guinea-pig virulence, H_2O_2 susceptibility, content of SAL and SL, presence of AI and TCH sensitivity. The first possibility was that there might be a causal relationship between one or more of the characteristics measured and, in particular, that SL might contribute to pathogenicity by preventing phagosome–lysosome fusion (Goren *et al.*, 1976). Since Western-type strains of phage types B and I have been found in the present study to be virulent in the guinea-pig but often to have low contents of SAL and SL, the possibility that SL is a major determinant of virulence now appears unlikely. On the other hand, the recently strengthened evidence that H_2O_2 generated by macrophages is responsible for killing intracellular mycobacteria (Jackett *et al.*, 1981; Walker and Lowrie, 1981) makes a causal relationship between virulence and H_2O_2 susceptibility far more likely.

The existence, in the present study, of a group of 8 Indian-type strains, which were attenuated but also resistant to H_2O_2 suggests that H_2O_2 production is not, however, the only mediator of host immunity. Of the 8 strains, the 6 whose lipids were examined all had AI present. It is interesting to speculate whether their attenuation might be due either to the presence of AI or perhaps to some deficiency, as yet unidentified, with which the synthesis of AI is linked. It is also notable that the laboratory strains H37Rv and H37Ra differed only in their lipid content; attenuation was accompanied by

substantial loss of SAL and SL, but not by increased H_2O_2 susceptibility or the appearance of AI (Table IV). These changes could well be characteristic of variation found only under laboratory conditions.

The second explanation proposed that the various associations with virulence are the result of parallel evolution among organisms that have been widely separated geographically and over long periods of time. Apart from the causal relationship between attenuation and H_2O_2 susceptibility, this now appears to be the most likely explanation for the remaining associations. The low virulence of the attenuated Indian-type strains is closely associated with the presence of AI, high susceptibility to H_2O_2 and phage type I. A slightly weaker association is evident between low virulence and an MIC of TCH of less than 8 mg/l, which is lower than with other strains of *M. tuberculosis* but higher than the MIC of 0.25–1 mg/l usual for bovine strains. Resistance to thiacetazone and to *p*-aminosalicylic acid were found to be even more weakly associated (Grange *et al.*, 1978).

There might be epidemiological reasons, such as an investigation of the efficacy of BCG vaccination in South East Asian countries (Indian Council for Medical Research/World Health Organisation, 1980), for wishing to identify South Indian attenuated strains using *in vitro* tests rather than expensive virulence tests. If the strains originated in India or nearby countries, the most informative tests would be detection of AI, phage typing, and determination of H_2O_2 susceptibility. However phage typing would be of much less value if the strains came from Western countries, since most of the Western-type phage I strains that we examined were fully virulent.

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