STUDIES ON THE VIRULENCE OF TUBERCLE BACILLI

Isolation and Biological Properties of a Constituent of Virulent Organisms

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Plate 12

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Virulent tubercle bacilli grow in the depth or on the surface of a liquid medium in a characteristic manner: they form tight bundles or "cords" consisting of bacilli in close parallel arrangement. The same is true for bacilli grown on the surface of solid media.

Although this pattern of growth had been described long ago (1), its significance was fully recognized only after its rediscovery by Middlebrook *et al.* (2). These authors compared the cultural behavior of strains of virulent and avirulent tubercle bacilli and found that the formation of cords is characteristic only for virulent strains and many BCG strains whereas the cells of most avirulent strains were not oriented and merely formed irregular clumps. For a large number of strains, cord formation and virulence were found to be correlated; the more virulent a strain, the tighter the cords it formed under suitable conditions (3). By addition of various substances to the culture medium, the tendency to form cords could be either weakened or enhanced. Thus, certain surface-active agents such as Tween 80 interfered considerably with the parallel arrangement of the bacilli (2, 4), whereas chick embryo extract has been shown to accentuate it (5).

Cord formation could be due to either physical or chemical factors. A strong polarity of the electric charge of the bacilli could account for their parallel arrangement. The existence of a differing electric charge in virulent and avirulent strains of tubercle bacilli has been reported (6). This does not exclude, however, the possibility of the existence of a chemical factor which causes the bacilli to adhere to one another and which could, in some way, be related to their virulence.

The part played by such a substance in the formation of cords could be explained by assuming that it is a secretion product of the growing bacilli which surrounds and covers the entire bacterial surface. After every cell division in either longitudinal or transverse plane, the daughter cells would automatically be held in parallel arrangement. Microscopic observations on growing young cultures can be cited in support of this genesis of cords (7, 8). While the exist-

ence of a specific substance surrounding or coating the surface of virulent tubercle bacilli has previously been postulated by Middlebrook and other authors (1, 9, 10) no morphological evidence has as yet been adduced for it. A recently described color reaction obtained only with virulent bacilli but not with avirulent variants (11) is also suggestive of differences in the chemical nature of their surface.

In previous experiments by the present author, characteristic effects of virulent tubercle bacilli on leukocytes were demonstrated (12). It was found that virulent bacilli, although phagocytized by polymorphonuclear leukocytes *in vivo*, were only slowly removed from peritoneal exudates; they seemed to injure the phagocytes and concurrently to multiply in the peritoneal cavity. Further work showed that the migration of leukocytes from blood or exudates *in vitro* was completely inhibited by virulent bacilli, whereas avirulent organisms did not affect the motility of the white cells (13).

On the basis of the facts cited, Dubos and Middlebrook and their associates have formulated the working hypothesis that there exists, in virulent bacilli, a chemical factor responsible for the formation of cords and also for the leukotoxic effect (3). The present study has revealed the presence, in virulent bacilli, of a material which appears to be responsible for cord formation and white cell injury. The isolation of this material and some of its properties will now be described in this paper.

EXPERIMENTAL

Disruption of the Cords of Virulent Tubercle Bacilli by Chemical Means.—It has long been known that tubercle bacilli can easily be suspended in paraffin oil. Personal observations with stained smears have shown that suspensions of wet virulent bacilli in mineral oil are no longer organized in typical cords after they have entered the oil phase, but are individually separated. These facts led to experiments devised to test whether paraffin oil or related solvents of the hydrocarbon series are capable of breaking up the bacillary cords in a selective way.

Following a technique used by Dr. Middlebrook in studies of the problem now under discussion,[1] a loopful of tubercle bacilli (strain H37Rv) from surface pellicles on Long's medium or from submerged cultures in Dubos' oleic acid–albumin medium was placed on a slide, covered with a coverslip, and the preparation placed under the microscope. Then, a drop of the solvent to be tested was placed on the edge of the coverslip. By capillary force, the solvent penetrated under the coverslip and its effect on the bacterial cords could be observed without the necessity of staining the bacilli.

As soon as reached by the oil, the cords entered the oil droplets and shortly after appeared disrupted. Only single, unoriented bacilli were found inside the

[1] Personal communication from Dr. Middlebrook.

droplets, whereas in the aqueous phase of the emulsion the cords remained unchanged. In Ziehl-Neelsen–stained smears of paraffin oil–treated bacilli, however, neither the morhology nor the tinctorial properties of the single bacterial cells showed any noticeable change.

The process of breaking up the cords could be accelerated by using warm paraffin oil, heating the preparation on a warm microscope stage, or by adding light paraffin oil (bayol F) or related compounds such as petroleum ether, pure hexane, or heptane; the lighter the solvent, the faster its action. Petroleum ether, *e.g.*, was able to break up cords within a few seconds. Figs. 1–5 show different stages of this action. At the end, the bacilli were no longer oriented, but lay helter-skelter and, on the basis of their arrangement alone, could no longer be recognized as belonging to a virulent strain (Fig. 6).

It could reasonably be assumed that the rapid disruption of the cords was due to a removal from the bacilli of a substance holding them in their characteristic parallel arrangement. The observation thus opened the way for an attempt to isolate this factor.

Extraction of Wet Tubercle Bacilli with Petroleum Ether[2].—Tubercle bacilli (strain H37Rv) were grown in 200 ml. Erlenmeyer flasks or in 1000 ml. Blake bottles on the surface of a modified Lockemann solution (14) or of the medium for surface growth developed by Dubos and Middlebrook (15). The media were inoculated with a loopful of a well grown surface culture and incubated at 37°C. The cultures were harvested when a thin veil of bacilli had just covered most of the surface of the medium, usually after 2 weeks. At this time, they were filtered through paper (Whatman No. 5) on a Büchner funnel. The bacilli were washed with distilled water, and, after draining on the filter for a few minutes, collected in a beaker. Approximately three volumes of petroleum ether (boiling point, 35 to 60°) was added, and the suspension was stirred with a magnetic stirrer for 3 minutes. After standing for 2 or 3 minutes, the supernatant fluid was decanted and the bacilli were extracted a second and third time in the same manner. The supernatant fluids were pooled, and centrifuged twice at 5000 R.P.M. for 60 minutes. The bacilli separated sharply, and the clear supernatant fluid was filtered through a Berkefeld and a Seitz filter. In contrast to the behavior of bacillary suspensions in paraffin oil (16), wet bacilli suspended in petroleum ether could be separated more readily from the liquid phase.

Nevertheless, the filtrate still contained a few acid-fast organisms. It was concentrated under reduced pressure in nitrogen atmosphere, again filtered through a Berkefeld filter, and centrifuged at 13,500 R.P.M. for 1 hour in the cold. The supernatant fluid was collected and evaporated. After evaporation of the solvent, a yellowish oily fluid remained which was dissolved in ethyl ether. Addition of ethanol in excess yielded a white precipitate. This was separated from the ethanol-ether mixture after standing overnight at 4°C. by centrifuging at 0°C. for 1 hour at 2500 R.P.M. The supernatant was discarded, the precipitate redissolved in ether, and again precipitated in alcohol and centrifuged in the cold. After this operation was repeated for a third time, the final precipitate was taken up in ethyl ether and dried *in vacuo* to constant weight.

[2] I am indebted to Dr. J. Freund for suggesting the methods of extraction described in the following paragraph.

Two gm. of bacilli (wet weight, after filtration and draining) yielded approximately 0.5 to 1 mg. of dry precipitate.[3] It should be emphasized that a similar precipitate could not be obtained from old cultures, or from bacilli which had been dried before the extraction procedures. Moreover, when fresh bacilli from either surface or depth cultures were dried on a slide at 37°C. petroleum ether no longer had an effect on the morphology of the cords. But the addition of a few drops of water, either before or after petroleum ether had been brought into contact with the bacilli, immediately restored the ability of the solvent to break up the cords. Thus, it can be assumed that under the conditions described above the substance which makes the bacilli adhere to each other is soluble in petroleum ether (or related compounds) only in the presence of water.

The extracted material is colorless, amorphous, and waxy. Its melting point is 30 to 31°C. At room temperature, it is easly soluble in ethyl ether, petroleum ether, paraffin oil, normal hexane and heptane, chloroform, benzene, dioxane; it is insoluble in ethanol, methanol, acetone, pyridine, water. It gives a negative biuret reaction and shows no fluorescence. Its elementary analysis is:

C 79.5 per cent, H 13.3 per cent, N. 0.6 per cent, P 0.0 per cent

In vacuo, it remains unchanged for months. When stained by the Ziehl-Neelsen technique, it is uniformly acid-alcohol–fast.

Effect of the Extracted Substance on Leukocytes in Vitro.—Since a characteristic effect of virulent bacilli on leukocytes had previously been demonstrated, it seemed interesting to see whether the extracted substance alone obtained from virulent bacilli had any similar action.

The technique for testing the effect of phagocytized bacilli on leukocyte migration has been described in detail previously (13). Leukocytes are allowed to phagocytize bacilli *in vitro* and are transferred thereafter on a plasma gel. After an incubation period of 16 to 20 hours, the width of the migration zone around the original inoculum is measured and the effect of the phagocytized bacilli on the locomotion of the cells can be measured quantitatively. In the present study, leukocytes from heparinized guinea pig blood were used. Preliminary phagocytosis experiments with the extracted substance alone were unsatisfactory since stable homogeneous emulsions of small droplets could not be prepared without the addition of emulsifiers. In an attempt to simulate the conditions existing in the earlier experiments with living tubercle bacilli, cells of avirulent tubercle bacilli (strain H37Ra) or of *B. subtilis* were coated with the extract from virulent bacilli by suspending approximately 0.01 gm. of dried bacilli in 5 ml. of a 0.01 per cent ether solution of the extracted material and evaporating the ether at 40°C. The dry bacilli were resuspended in saline containing 0.5 per cent bovine al-

[3] Ziehl-Neelsen–stained smears of the precipitate were carefully checked for the presence of acid-fast microorganisms. In some instances, a few poorly stained "ghosts" or acid-fast granules were found. Since no immunological work was involved in the present study, no attempt was made to remove these occasional traces of bacillary residues. The tuberculin tests reported later show that they were insignificant.

bumin. Controls were prepared by treating the bacilli with ether alone and suspending them in the same way. The bacilli were then allowed to be phagocytized and the leukocytes tested for migration as described previously. The results were read after 16 to 20 hours' incubation and the intensity of migration expressed according to an arbitrary scale ranging from 0 to ++++.

A typical experiment is summarized in Table I. It will be seen that cells of *B. subtilis* and of avirulent tubercle bacilli coated with the material extracted from virulent tubercle bacilli act upon leukocytes which have engulfed them in the same way as do virulent bacilli; *i.e.*, the migration of the cells is inhibited.

TABLE I

The Effect of Avirulent Tubercle Bacilli (Strain H37Ra) and of B. subtilis Coated with Extract from the Virulent Strain H37Rv, on the Migration of Leukocytes

Leukocytes after phagocytosis of:	Intensity of migration
Avirulent tubercle bacilli, coated with extract	0
B. subtilis coated with extract	+
Controls:	
Ether-extracted avirulent tubercle bacilli	++++
Ether-extracted *B. subtilis*	++++
Untreated avirulent tubercle bacilli	+++
Untreated *B. subtilis*	++++
Untreated virulent tubercle bacilli	0
Leukocytes without engulfed bacilli (control)	++++

The intensity of leukocyte migration is expressed according to an arbitrary scale, ranging from 0 (no migration) to ++++ (maximum migration).

This effect seems to be due to the coating, since uncoated bacilli have no effect on the locomotion of the cells. On the other hand, virulent bacilli extracted with petroleum ether no longer inhibit leukocyte migration, as can be seen from Table II.

Hence, disrupting the bacillary cords of virulent tubercle bacilli by treatment with petroleum ether was accompanied by the removal from the bacilli of a material which, by itself, simulated the migration-inhibitory effect of the untreated virulent organisms, whereas the virulent bacilli, for their part, lost their inhibitory action by the removal of this material. This material, therefore, will be referred to hereafter as "cord factor."

Toxicity of the Cord Factor for Mice.—In the experimental series reported hereafter, the effects of parenteral injections of cord factor into mice of four dif-

ferent strains were studied. The following four strains of mice were used: Swiss albino, CFI, C57 black, and dba. They were chosen because they were known to have different degrees of susceptibliity to tuberculous infections (17).

TABLE II

The Effect of Tubercle Bacilli, Extracted with Petroleum Ether, on the Migration of Leukocytes

Leukocytes after phagocytosis of:	Intensity of migration
Virulent tubercle bacilli, extracted with petroleum ether	+++
Avirulent tubercle bacilli, extracted with petroleum ether	+++
Controls:	
Virulent tubercle bacilli, untreated	0
Avirulent tubercle bacilli, untreated	++++
Control for leukocyte migration	++++

Symbols as in Table I.

TABLE III

The Effect of Injections of Cord Factor Obtained from Virulent Tubercle Bacilli (Strain H37Rv) upon Different Strains of Mice

Mouse strain	Suscepti-bility to in-fection with tubercle bacilli H37Rv	No. of mice in each group	No. of intra-perito-neal injec-tions	Interval between the injec-tions	Cord factor injected per dose	Results
				days	*mg.*	
Swiss albino	Low	8	1	—	20	No toxic manifestations
Swiss albino	Low	25	12	2 to 3	0.1	No toxic manifestations
CFI	Low	5	1	—	20	Slight loss of weight after injection
CFI	Low	25	12	2 to 3	0.1	No toxic manifestations
C57 black	High	5	1	—	20	Slight loss of weight after injection
C57 black	High	15	3 to 8	2 to 3	0.1	D9, D9, D9, D9, D9, D9, D10, D11, D13, D13, D13, D18, D19, S25, S25
C57 black	High	15	3 to 8	2 to 3	0.02	D9, D9, D9, D10, D12, D12, D12, D15, D17, D21, S25, S25, S25, S25, S25
Dba	Very high	3	1	—	20	No toxic manifestations
Dba	Very high	7	3 to 6	2 to 3	0.1	D9, D9, D9, D11, D13, D13, D19
Dba	Very high	5	3 to 7	2 to 3	0.02	D9, D11, D12, D14, S25

D followed by a number indicates death of the mouse and the number of days of survival after the first injection. S denotes that the mouse survived and was sacrificed after the number of days indicated. The Swiss mice used in this experiment were of the Webster strain obtained from Tumblebrook Farms, Brant Lake, New York. The CFI, C57, and dba strains came from Carworth Farms, New City, New York.

Acute toxicity tests: Amounts up to 20 mg. of cord factor, dissolved in 0.1 ml. of light paraffin oil, were injected intraperitoneally or subcutaneously into mice. The same amounts of cord factor, dissolved in propylene glycol, were used for intravenous injections. Chronic toxicity tests: The doses used amounted to 0.02 mg. or 0.1 mg. likewise dissolved in 0.1 ml. of light paraffin oil and repeatedly injected subcutaneously or intraperitoneally at 2 to 3 day intervals. The control mice were injected at identical intervals with 0.1 ml. of plain paraffin

oil, and in one series of experiments reported below, with extracts from sources other than virulent tubercle bacilli. All animals were housed in groups of 5 mice per cage and kept on a diet of pellets and water. Numerous toxicity tests were carried out between the months of March and August, 1949. Over this period of time, a total of 96 mice was used for acute toxicity and 517 for chronic toxicity tests; 131 mice, injected with plain paraffin oil, served as controls.

Table III summarizes typical experiments of this series. Two findings stand out:

1. The cord factor is apparently non-toxic after a single intraperitoneal injection of as much as 20 mg.; there were no demonstrable toxic manifestations in any of the four strains of mice used. But after a few repeated injections, even in amounts of only 0.02 mg. per dose, death occurred in the majority of animals of some of the strains. The control mice injected with paraffin oil alone never died from this treatment nor did they show comparable toxic manifestations. When these mice were killed, peritoneal exudates and signs of peritonitis were found at autopsy.

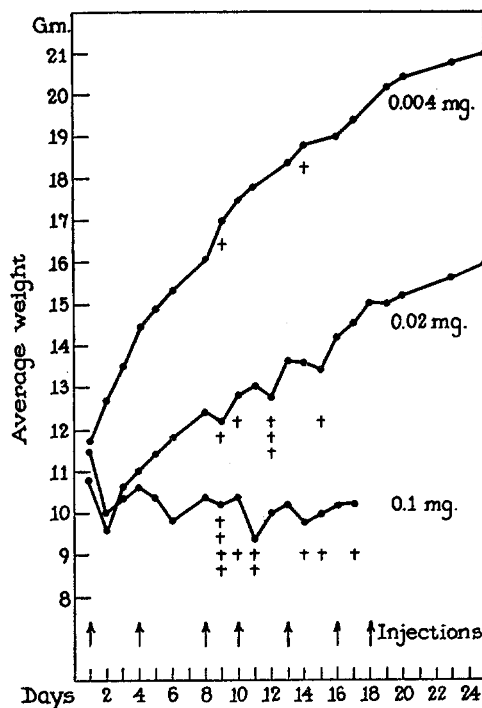
2. The susceptibility of the four strains of mice to the toxic effect of repeated injections of the cord factor paralleled their different susceptibility to infection with the corresponding strain of living bacilli. The results after intravenous and subcutaneous injections of cord factor were essentially identical with those described in Table III.

The response of susceptible mice to the toxic effect of repeated injections was characterized by a progressive loss of weight. The mice appeared sick and emaciated and began to die from the 9th day on. A typical weight and survival curve is shown in Text-fig. 1. At autopsy, the animals showed peritoneal exudates, and, more characteristically, severe hemorrhages of the lungs. Every lobe was uniformly congested and deep red. Similar pulmonary changes were never observed in control animals killed at the same time. Other organs showed no striking gross pathological changes. A more detailed study of these lesions will be published later.

Within certain limits, the action of the cord factor depends upon the dosage used, but death of the mice has never occurred earlier than 9 days after the first injection (see Text-fig. 1). Practically identical results were obtained, when susceptible mice were given a single intraperitoneal injection of 0.5 mg. and, after 9 days, a second dose of 0.05 mg. The death rate of the mice was the same as with repeated injections of small doses, as described above.

The Toxicity of Petroleum Ether Extracts of Various Origin on Leukocytes and Mice.—All the experiments reported thus far were carried out with a preparation of cord factor obtained from cultures of the virulent strain H37Rv. In order to test the specificity of its action, similar extracts were prepared from the following five strains of *Mycobacteria:* Avirulent variant of human tubercle bacilli, strain H37Ra; virulent bovine tubercle bacilli, strain Vallée; attenuated BCG bacilli, strain Phipps; smegma bacilli, strain Basel; *M. phlei*, strain

Basel. Furthermore, fresh wet homogenates of ox lungs and brain were extracted in the same manner with petroleum ether. The yields of the material obtained from the H37Ra, the *M. phlei*, and the BCG strains were very small compared to the ones obtained from virulent strains, whereas smegma bacilli gave an abundant precipitate. The fractions differed somewhat in their solubility and melting points, but in the absence of definite criteria for purity these



TEXT-FIG. 1. Average weights of three groups of ten C57 mice each, injected with 0.004 mg., 0.02 mg., and 0.1 mg. cord factor respectively. The mice were injected at the days indicated by an arrow. Each cross (+) represents one dead mouse.

variations do not necessarily indicate chemical differences. Beside the cord factor from the H37Rv strain, only the factor obtained from the virulent bovine strain Vallée was solidly acid-fast.

The five bacillary extracts were tested for their action on leukocyte migration by using them as a coating for organisms of *B. subtilis*. The results are summarized in Table IV. It will be seen that only the extract of the virulent bovine strain Vallée exerted an inhibitory action comparable in intensity with that of the cord factor obtained from the H37Rv strain. Considerable inhibition of migration occurred also with an extract obtained from BCG bacilli, whereas

those obtained from *M. phlei*, from smegma bacilli, and from the avirulent strain H37Ra were devoid of any inhibitory action. Thus, the varying degrees of pathogenicity of the five strains for the guinea pig were paralleled to some extent by variations in the activity of the substances extracted from them to inhibit migration. Extracts from BCG bacilli inhibited the migration of leukocytes to a similar extent as did living organisms of this strain (18).

In addition, the susceptibility of two different strains of mice, C57 and CFI, to the toxic action of the different extracts was studied. These two strains were chosen because previous experiments (18) had revealed a difference in their susceptibility to infections with different strains of tubercle bacilli. Whereas the C57 strain of mice was as susceptible to the human strains H37Rv and Jamaica No. 22 as to the bovine strain Ravenel and Vallée, the CFI

TABLE IV

The Effect of Phagocytized B. subtilis, Coated with Extracts from Different Mycobacteria, on the Migration of Leukocytes

Leukocytes with engulfed cells of *B. subtilis* coated with extracts from:	Intensity of migration
Avirulent tubercle bacilli (strain H37Ra)	++++
Virulent tubercle bacilli (strain H37Rv)	0
Virulent tubercle bacilli (strain Vallée)	0
Smegma bacilli (strain Basel)	+++
M. phlei (strain Basel)	++++
BCG (strain Phipps)	+
Leukocytes without engulfed bacilli (control)	++++

Symbols as in Table I.

mice were considerably more susceptible to infections with the two bovine than with the two human strains. It is in good agreement with these findings that the CFI mice were not susceptible to the toxic action of the cord factor from H37Rv bacilli, whereas the extract obtained from the bovine strain Vallée was toxic for these mice as well as for the C57 strain (Table V).

On the other hand, an extract obtained from smegma bacilli and from BCG bacilli was toxic for both strains of mice. In this case, there was no parallelism between toxicity of the extract and the action of living bacilli. The observation may have some bearing on earlier findings concerning the toxicity of dead saprophytic *Mycobacteria* suspended in mineral oil (19, 20). A similar toxicity was observed with extracts from ox lungs, an entirely unrelated source. While the findings in animals dying after injections of extract from smegma bacilli or ox lungs were essentially identical with those described for the effect of cord factor, it is noteworthy that in some animals death occurred as early as 5 days after the first dose, while 9 days was the shortest survival time

observed after injection of cord factor from virulent tubercle bacilli. Thus, toxicity of foreign lipids, *per se*, may be completely non-specific.[4]

It will further be noted from Table V that the substance extracted from the avirulent variant of the H37 strain (H37Ra) was devoid of any toxicity for both strains of mice. This fact seems important; it makes the possession of

TABLE V

The Effect of Repeated Intraperitoneal Injections of Petroleum Ether Extracts from Different Strains of Mycobacteria in Mice

Strain of mice	No. of mice	Petroleum ether extracts from:	Extract injected per dose	No. of injections (at 2 to 3 day intervals)	Results
			mg.		
C57 black	10	H37Rv	0.1	3 to 8	D9, D9, D10, D10, D10, D13, D14, D16, D19, S25
" "	10	Vallée	0.1	3 to 6	D9, D9, D10, D10, D10, D11, D11, D14, D15, D18
" "	10	BCG	0.1	3 to 8	D9, D11, D11, D14, D17, S25, S25, S25, S25, S25
" "	10	H37Ra	0.1	8	No toxic manifestations
" "	10	Smegma	0.1	2 to 8	D6, D6, D8, D8, D10, D18, S25, S25, S25, S25
" "	10	*Phlei*	0.1	8	No toxic manifestations
" "	10	Ox brain	0.1	8	No toxic manifestations
" "	8	Ox lungs	0.1	2 to 7	D5, D6, D6, D10, D13, D15, D18, S25
CFI	10	H37Rv	0.1	8	No toxic manifestations
"	10	Vallée	0.1	3 to 8	D9, D10, D10, D13, D18, D19, D20, S25, S25, S25
"	10	BCG	0.1	4 to 8	D13, D15, D16, D23, S25, S25, S25, S25, S25, S25
"	10	H37Ra	0.1	8	No toxic manifestations
"	10	Smegma	0.1	2 to 8	D5, D7, D8, D14, D14, D23, S25, S25, S25, S25
"	10	*Phlei*	0.1	8	No toxic manifestations

Symbols as in Table III.

a toxic component a point of major difference between the two variants. Equally atoxic were extracts from *M. phlei*, whereas the toxicity of the material obtained from BCG bacilli does not parallel the lack of virulence of these organisms *in vivo*.

[4] With regard to the toxicity of the material obtained from cultures of BCG bacilli, it has to be kept in mind that a given amount of toxic material is derived from a much greater number of organisms than in the case of the two virulent strains.

Relationship between Cord Factor and Tuberculin Activity.—Previously infected tuberculin-positive guinea pigs were injected intradermally with 0.05 ml. of paraffin oil containing 0.5 mg. of cord factor and simultaneously, into another skin area, with the same amount of old tuberculin 1:10. The resulting reactions are shown in Table VI.

In another series, normal guinea pigs were given three intraperitoneal doses at 3 day intervals each containing 2 mg. of cord factor in paraffin oil. They were tested 13 days after the last injection by intradermal injections of the same amounts of old tuberculin as used in the above experiment. The response to the injections was negative in all instances.

TABLE VI

Skin Reactions of Normal and Tuberculous Guinea Pigs to Intracutaneous Injections of Cord Factor (0.5 Mg. in 0.05 Ml. Paraffin Oil)

Guinea pig	Material injected	Reactions after		
		24 hrs.	48 hrs.	72 hrs.
26-59	Old tuberculin 1:10	++	+++ N	+++ N
	Cord factor	+	(+)	0
45-60	Old tuberculin 1:10	+++	+++ N	+++ N
	Cord factor	++	+	0
	Paraffin oil	+	(+)	0
1-72	Old tuberculin 1:10	+	(+)	0
	Cord factor	++	+	(+)
	Paraffin oil	+	0	0

The intensity of erythema and swelling is indicated by the symbols 0 to +++; N means central necrosis.

The guinea pigs 26-59 and 45-60 had been injected previously with virulent tubercle bacilli (H37Rv); No. 1-72 was a normal guinea pig.

Cord factor thus does not contain tuberculin or functionally related substances in a reactive form, nor does it give rise to local or systemic reactivity with tuberculin.

The Significance of the Cord Factor for the Viability and Pathogenicity of Virulent Tubercle Bacilli.— Filtered and washed bacilli of young surface cultures of two strains, H37Rv and Vallée, were each divided into two portions. One of them (A) was extracted with petroleum ether in the manner described; after centrifugation the supernatant was discarded and the bacilli resuspended in fresh petroleum ether. The other portion (B) was washed, slightly ground in a mortar, and resuspended in saline; the coarse particles were allowed to settle and the supernatant brought to the same optical density as the bacterial suspension in petroleum ether. Serial dilutions of both suspensions were now

prepared, portion A in petroleum ether, portion B in saline. From each dilution subcultures were made on oleic acid–albumin plates and in Tween-albumin tubes. The results are reported in Table VII. It will be seen that extracting the bacilli with petroleum ether in the manner described did not kill them. On proper media, extracted organisms grew out in approximately the same number as unextracted controls. Furthermore, the morphology of the colonies formed on oleic acid–albumin agar inoculated with extracted and unextracted bacilli was identical. Both presented the typical aspect of cord-forming bacilli.

TABLE VII

The Growth of Virulent Tubercle Bacilli after Extraction with Petroleum Ether

Strain	Dilution of the original suspension	Growth after 4 wks.			
		Unextracted bacilli suspended in saline		Bacilli extracted with and suspended in petroleum ether	
		Agar plates	Fluid medium	Agar plates	Fluid medium
H37Rv	10^{-1}	++++	Growth	++++	Growth
	10^{-3}	++++	Growth	++++	Growth
	10^{-5}	+++	Growth	++	Growth
	10^{-6}	102 colonies	Growth	36 colonies	Growth
	10^{-7}	23 colonies	No growth	12 colonies	Growth
	10^{-8}	No growth	No growth	4 colonies	No growth
	10^{-9}	No growth	No growth	No growth	No growth
Vallée	10^{-1}	++++	Growth	++++	Growth
	10^{-3}	++++	Growth	++++	Growth
	10^{-5}	++	Growth	98 colonies	Growth
	10^{-6}	12 colonies	Growth	3 colonies	No growth
	10^{-7}	3 colonies	No growth	No growth	No growth
	10^{-8}	No growth	No growth	No growth	No growth

The growth intensity on agar plates is indicated by the symbols + to ++++ or by the number of colonies, if they were not too numerous to be counted. In the fluid media, no attempt was made to quantify the growth.

The only difference between the two series was a faster initial growth rate of the unextracted bacilli. Colonies appeared somewhat earlier on the plates inoculated with bacilli of portion B.

The slower initial growth rate of the petroleum ether series may well have been due to the finer grade of dispersion of these bacilli. Microscopic observation of the plates during the first days of incubation showed that almost all colonies grew out from single bacterial cells, whereas in the saline series they developed mostly from small clumps of bacilli. The growth of these colonies therefore became apparent relatively earlier. Similar results were obtained when the petroleum ether–extracted bacilli were resuspended in saline and the serial dilutions made in saline instead of petroleum ether.

A few guinea pigs were infected with bacilli (strain H37Rv) from subcultures of extracted organisms. There appeared to be no difference in virulence between these bacteria and subcultures from unextracted organisms of the same strain and age.

In another experiment, tests were made to determine whether prolonged contact with petroleum ether would injure the bacilli. A suspension of virulent tubercle bacilli in petroleum ether was stored in the ice box and subcultures on oleic acid–albumin plates were made daily for 2 weeks; after an incubation period of 15 days, each plate was removed from the incubator and stored in the cold until all plates could be counted simultaneously. There was no significant difference in the growth between the first and any of the subsequent

TABLE VIII

Evidence of Gross Tuberculous Lesions in Guinea Pigs Injected with Extracted Tubercle Bacilli, Strain H37Rv

Guinea pig No.	Gross tuberculous lesions of:				
	Omentum	Portal lymph nodes	Spleen	Liver	Lungs
1	+	+	+	+	–
2	+	+	+	–	+
3	+	+	E	–	–
4	+	–	E	–	–
5	–	–	–	–	–
6	–	–	–	–	–

+ indicates that the organ showed nodules containing acid-fast bacilli. – indicates that the organ showed no macroscopic pathological signs. E indicates that the spleen was enlarged but contained no gross nodules and smears were negative for acid-fast bacilli.

plates. Even after 4 weeks of contact with petroleum ether at 4°C., bacilli grew out on agar plates.

While it was clear that even prolonged exposure of virulent tubercle bacilli to petroleum ether failed to reduce their ability to grow on suitable culture media, it remained to determine whether the pathogenicity of the extracted cells was affected. To extracted and unextracted bacilli (strain H37Rv) sufficient saline was added to make suspensions of a density corresponding to approximately 2×10^6 bacilli per ml. Of these suspensions, mice (strain C57) were given 0.1 ml. intravenously.

16 mice were injected with unextracted bacilli. They all died from tuberculosis, showing extensive lung lesions at autopsy. The average survival time of this group was 25.1 days (range: 20 to 44). During this period, the 15 mice injected with extracted bacilli showed no signs of illness, even after an additional period of 2 weeks. They all gained weight. 60 days after inoculation the mice of this group were sacrificed. No gross tuberculous lesions could be

TABLE IX

Evidence of Gross Tuberculous Lesions in Guinea Pigs Injected with Extracted and Unextracted Tubercle Bacilli, Strain H37Rv

Guinea pigs injected with	Dilution of the bacillary suspension	Survival time	Degree of tuberculous processes
		days	
Unextracted bacilli	Undiluted	20	IV
		21	III
		25	IV
		26	V
		26	V
	1:20	19	II*
		26	IV
		32	V
		39	V
		42	V
	1:400	37	I*
		45	IV
		57	III
		66	V
		70S	V
Extracted bacilli	Undiluted	18	0*
		18	I*
		27	IV
		28	IV
		32	V
	1:20	32	IV
		37	V
		52	IV
		70S	II
		70S	I
	1:400	37*	I
		70S	III
		70S	0
		70S	0
		70S	0

The degree of the tuberculous processes found at autopsy is expressed as follows (24):—

0, no gross tuberculous changes.

I, nodules in the omentum.

II, nodules in the omentum and portal lymph nodes.

III, nodules in omentum, portal lymph nodes, and spleen.

IV, moderate generalized tuberculosis.

V, pronounced generalized tuberculosis.

A number followed by S indicates the day after inoculation at which the animal was sacrificed.

* Indicates that the animal died of an intercurrent infection other than tuberculosis.

detected in the lungs of these animals; the spleens were of normal size. Smears of the lungs, however, showed acid-fast bacilli in 9 out of 15 mice.

A similar experiment was performed, using guinea pigs. Six animals were given intraperitoneally approximately 10^6 unextracted bacilli, another group of six was injected with the same amount of extracted organisms. The results were in agreement with the mouse experiment. The controls died after an average of 27.6 days (range: 22 to 34). All had signs of a generalized tuberculosis. The animals of the group injected with extracted bacilli gained weight during this time. They were sacrificed 50 days after inoculation. The findings at autopsy are reported in Table VIII. It shows that there is a difference in the development of the disease in the two groups. Only 2 guinea pigs had a generalized tuberculosis, 2 had a moderate disease, and 2 showed no gross lesions.

The experiment was repeated on a larger scale using graded doses. The guinea pigs were inoculated by the intraperitoneal route with 1.0 ml. of a bacillary suspension diluted as indicated in Table IX. The original suspensions contained approximately 10^8 bacilli per ml. (Table IX).

It appears from these experiments that bacilli devoid of the cord factor are still pathogenic, but that in some of the guinea pigs the disease developed considerably more slowly when the animals were inoculated with comparable amounts of extracted bacilli.

All guinea pigs were tested for tuberculin reactivity 3 weeks after inoculation; all were positive. No attempt was made to isolate tubercle bacilli from the organs of the sacrificed animals which showed no gross evidence of tuberculous lesions. The fate of the injected bacilli which produced no disease or only minimal lesions will have to be studied in further experiments.

DISCUSSION

The experiments reported in this paper were based on the working hypothesis formulated by Dubos and Middlebrook and their associates that a substance produced by virulent tubercle bacilli causes the organisms to adhere to each other and thus to form the characteristic cords, and that this substance by itself would be responsible for some of the characteristic properties of virulent organisms (3). It was further assumed that this hypothetical substance was located at the surface of the bacterial cell, and that it could be removed without using harsh methods of extraction.

The ease with which solvents of the hydrocarbon series were found to disrupt the cords of virulent tubercle bacilli finds its simplest explanation in a rapid dissolution of a substance (or of substances) coating the bacillary surface. This coating appears lipoidal in nature and is secreted by the bacillus. It will not go into solution as long as the bacilli are in an aqueous surrounding. There

they form cords. In a lipophilic phase, however, it becomes separated from the microorganisms and goes into solution. This results in a suspension of single bacterial cells or small bacterial aggregates; the better the solvent, the finer the suspension of the bacteria. Hence, bacilli in petroleum ether are strictly single cell suspensions. Bacilli grown in fluid culture media containing Tween 80 form diffuse suspensions consisting of single cells or of small clumps of bacilli. Since Tween 80 renders the medium more lipophilic, it causes wetting of the bacillary surface thus interfering with the bacilli adhering to each other and forming cords. Moreover, part of the cord factor produced by the bacilli may go into solution continuously. Similar differences have been observed in the growth pattern of the bacilli in pathological specimens from tuberculous patients or animals. While it is common to find tight bacillary cords in aqueous environments such as urine or exudates, cords are often lacking in tissues and cell fluids.

Two distinct and characteristic properties of the isolated cord factor have been described: its systemic toxicity and its ability to inhibit migration of leukocytes. The action of the agent seems to affect chiefly the blood vessels, especially the capillaries, but the mode of action is not yet clear. The fact that a single high dose was without effect, and that toxic manifestations appeared only after repeated injections, and the observation that the mice did not die until the 9th day after the first injection, regardless of the dosage used, suggests some kind of sensitization whereby the cord factor is able to exert its toxic action on tissues only after preparatory injections.

The possibility of stirring up a latent virus infection by injecting biological material into mice may be considered. In the present study, no attempt was made to cultivate an infectious agent from hemorrhagic mouse lungs. But the following points render a virus etiology of the observed lesions not very likely: uniform results were obtained over a period of 6 months during which time a relatively high number of animals from different shipments was used; the mice were all caged in small groups which makes conditions unfavorable for the occurrence of cross-infections; the control animals injected with paraffin oil or petroleum ether extracts from other sources never developed similar lesions (except for the extracts from smegma and BCG bacilli and from ox lungs mentioned in the text); finally the fact that CFI mice were susceptible only to extracts from organisms of the Vallée strain which suggests a specific action of the toxic factor. If therefore, an unknown infectious agent participates in producing the described lesions it would only seem to be activated by specific agents and under closely defined conditions.

The action of the cord factor on leukocytes is more readily understandable. The substance is both leukotactic and leukotoxic, and its effects resemble so closely those produced by virulent bacilli that it can be regarded as being responsible for their action on leukocytes. This view is further supported

by the fact that extracted bacilli free of cord factor no longer inhibit leukocyte migration.

It is important to recall that bacilli devoid of the cord factor are still able to grow in culture media. Thus, the cord factor seems not to be essential for multiplication *in vitro* although it is continuously synthesized by the bacilli. Multiplication of the bacilli within the host may depend upon its presence insofar as it will protect the bacilli from being inactivated by the phagocytes. The infection experiments with extracted but living bacilli can be interpreted in this sense. If the naked bacilli are phagocytized before they have a chance to be coated with a new layer of cord factor, they are destroyed by the phagocytes. If, however, they escape immediate engulfment by the host leukocytes, they can form a new protective layer of cord factor and from then on they will act as unextracted bacilli would. This may be the case if the injected bacilli outnumber the phagocytic cells immediately available. The smaller the infective dose the fewer bacilli will escape phagocytosis within the first hours of infection. This interpretation is based entirely on the assumption that avirulent bacilli are inactivated or killed by phagocytes whereas virulent organisms are not. There is no direct evidence to support the view that polymorphonuclear leukocytes are able to kill avirulent tubercle bacilli or to interfere with their multiplication, although some earlier observations may be interpreted in this sense (12, 21, 22). On the other hand, the injury to the leukocytes caused by some strains of virulent bacilli seems to be well established (13, 23). This action can now be attributed to a constituent which has been separated from virulent tubercle bacilli, and which, by itself and in the absence of bacilli, exerts a certain effect on leukocytes.

A suggestive comparison can be made of the rôle played by this constituent in the pathogenicity of the tubercle bacillus—especially in the non-immune host—and the function of the capsule polysaccharide in pneumococcus infections. Neither tubercle bacilli nor pneumococci are killed when the surrounding layer is removed from the bacillary cell *in vitro*. But the naked organisms are no longer protected against the action of the phagocytes of a susceptible host.

The fact that in the same group of susceptible guinea pigs (Tables VIII and IX) some animals developed a progressive disease within a much shorter time than others, seems to support this view. The extracted bacilli escaping immediate phagocytosis will act like freshly injected normal virulent organisms, the others are inactivated. This interpretation is based on the experimental evidence that the animals injected with extracted bacilli were given amounts of living organisms approximately equal to those injected into the control animals which received unextracted bacilli. Plate counts were made to determine the number of injected organisms. While the inaccuracy of such counts has to be kept in mind, the results summarized in Tables VIII and IX may

nevertheless be due to factors lying beyond the limits of error of this method. It should be emphasized, however, that there is no evidence to suggest that the possession of cord factor is the only difference between virulent and avirulent strains of tubercle bacilli.

The fact that the viability of virulent tubercle bacilli may be less affected by chemical treatment than their pathogenicity, is known from earlier observations. Thus, after treatment with sodium hydroxide a larger proportion of organisms is able to grow on artificial media than can multiply in a susceptible host (24). However, the treatment with sodium hydroxide kills a considerable number of bacilli (25) beside reducing the pathogenicity of the survivors, whereas, there is no indication that compounds of the paraffin oil series exert a similar killing effect on the organisms. A reduced degree of virulence of bacilli suspended in paraffin oil has been reported earlier (26, 27), but it is not clear from these papers whether part of the bacilli injected were not dead.

The question arises as to whether a similar or identical fraction has been previously isolated from tubercle bacilli. The list of compounds described by Anderson and his coworkers (28) contains no such substance. However, we are at present, probably not dealing with a pure compound. One would expect only cord-forming organisms to yield an appreciable amount of extract, provided the latter consisted of the cord substance alone. Indeed, the yields obtained from the attenuated BCG bacilli and the avirulent strain H37Ra were very small compared with the amounts extracted from the virulent strains. On the other hand, the non-cord-forming smegma bacilli yielded large amounts of material soluble in petroleum ether. It is most likely that by this method of extraction, in most cases, mixtures containing the active principle in varying proportions are obtained. The chemical composition of the cord factor is now under investigation.

The biological differences of the cord factors obtained from various human and bovine strains of tubercle bacilli suggest that these factors are chemically different. Further studies extended over a larger number of strains will have to disclose whether the differences are type- or strain-specific.

In this study, the cord factor extracted from two virulent strains was acid-fast. According to the literature, the only acid-fast substance obtained from *Mycobacteria* is mycolic acid (29), but since the chemical properties of the cord factor differ from those described for mycolic acid, it must be assumed that the present preparation of cord factor is either a crude mixture contaminated with mycolic acid or a new, as yet unknown, acid-fast lipid of the tubercle bacillus. Likewise, the relation of the cord factor to the agents extracted by Choucroun (30) has not yet been established.

One fact remains to be recalled in this connection: as yet, most of the work on the chemistry of tubercle bacilli was done by extracting dried, heat-killed

bacilli from old surface cultures. By the method of extraction used in the present experiments, no appreciable amount of cord factor was obtained from dried or heat-killed bacilli. Likewise, the age of the organisms seemed to be important; best yields were obtained from relatively young cultures. Since the cord factor represents only a small fraction of the total bacillary mass, its presence could have been masked in earlier work by the large amounts of lipids obtained by more exhaustive procedures of extraction.

Mention should finally be made of the possible significance of the toxicity of the cord factor in the course of a tuberculous infection. Although concentrations as high as those applied in these experiments probably do not occur in natural infection, it can be assumed that a continuous small output of the substance is constantly being supplied to the surrounding tissue by growing bacilli. The vascular changes which occur after injections of cord factor may have some bearing on recent work reemphasizing the fact that pathological alterations of small blood vessels at the site of tuberculous lesions are early manifestations of the infection (31, 32). Moreover, experimental conditions have long been known in which a considerable amount of cord factor enters the circulation of an animal: the instances where large masses of living or dead bacilli are injected along with paraffin oil. Since paraffin oil dissolves the cord factor, the resulting effect may, to some extent, be compared to the one obtained by direct injections of the isolated substance. Beside extensive tuberculous lesions produced by this procedure, toxic effects are reported which manifest themselves by vascular changes in the lungs not unlike the changes initiated by injections of cord factor (33, 34).

SUMMARY AND CONCLUSIONS

The bacillary cords characteristic for virulent tubercle bacilli are readily disrupted when wet bacilli are suspended in hydrocarbons such as paraffin oil or petroleum ether.

The disruption of cords is due to the removal of a material coating the surface of the bacilli and causing them to adhere to each other.

This material can be obtained from virulent bacilli by extracting them with petroleum ether. It is a lipid. Avirulent variants of tubercle bacilli do not yield a similar material after extraction in the same manner; only little of it is obtained from BCG bacilli.

The following properties of the fraction obtained by petroleum ether extraction are described:

(*a*) It inhibits the migration of leukocytes *in vitro*.

(*b*) If repeatedly injected in small doses into mice, it is toxic, whereas a single high dose does not give rise to toxic manifestations.

(*c*) The susceptibility of mice to the toxic action of repeated injections paral-

lels to some extent their degree of susceptibility to infection with the strain of tubercle bacilli from which the fraction was obtained.

(*d*) The injection of the extracted material into guinea pigs does not induce a state of allergic reactivity toward tuberculin.

Likewise, tuberculin-positive guinea pigs do not show hypersensitivity against injections of the extracted substance.

Bacilli extracted with petroleum ether do not lose their viability. They grow out normally *in vitro*, and they are still pathogenic. However, the removal of the petroleum ether-soluble lipid from the bacilli results in a loss of the ability of the organisms to inhibit the migration of polymorphonuclear leukocytes. Moreover, mice and guinea pigs infected with extracted bacilli may develop tuberculosis considerably slower than animals injected with comparable amounts of unextracted organisms.

The significance of these findings is discussed in relation to the problem of the virulence of tubercle bacilli.

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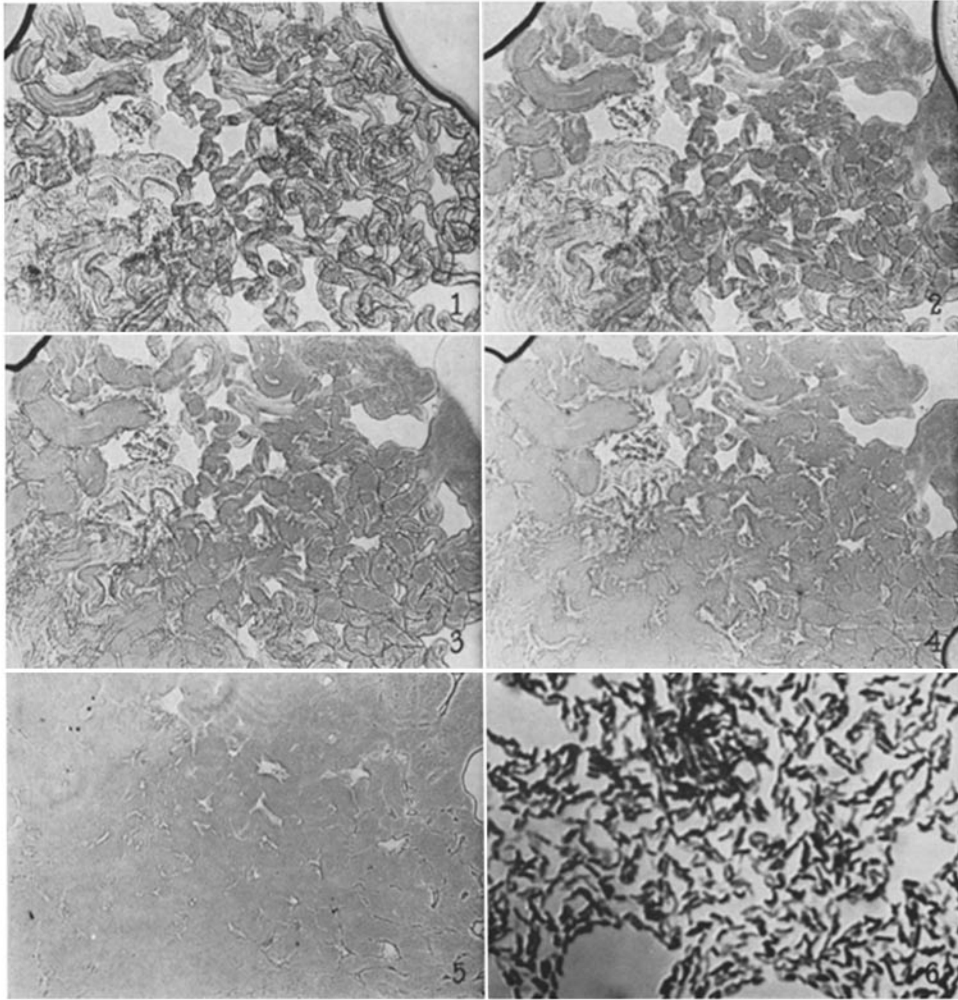
EXPLANATION OF PLATE 12

The photographs were made by Mr. Julian Carlile.

FIGS. 1–5. The photographs demonstrate the action of petroleum ether on a small pellicle from a surface culture of the human strain of tubercle bacilli H37Rv (virulent). Serial pictures of the same field were taken at short intervals; they show different stages of disintegration undergone by the bacillary cords. The original shape of the pellicle can be recognized throughout the entire series, but in the later stages the bacilli are no longer adhering to each other. × 62.

FIG. 6. Ziehl-Neelsen–stained tubercle bacilli H37Rv (virulent) after the action of petroleum ether on the bacillary cord. This photograph stems from the same preparation as the one shown in Figs. 1–5. The coverslip was removed and the bacilli stained after evaporation of the petroleum ether. × 1100.



(Bloch: Virulence of tubercle bacilli)