STUDIES ON THE VIRULENCE OF TUBERCLE BACILLI

VARIATIONS IN VIRULENCE EFFECTED BY TWEEN 80 AND THIOSEMICARBAZONE

BY HUBERT BLOCH, M.D., AND HANS NOLL, PH.D.

(From the Division of Applied Immunology, The Public Health Research Institute of The City of New York, Inc., New York)

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In 1947, Middlebrook, Dubos, and Pierce (1) pointed out that the organisms in colonies of virulent tubercle bacilli are arranged in parallel bundles or "cords" while avirulent bacilli have a random orientation. On the basis of these observations, Dubos (2) postulated the existence of a surface constituent in virulent organisms which was responsible for this type of growth and for their pathogenicity. Subsequent studies from this laboratory showed (3) that mild petrol-ether extraction of young cultures removed a material from virulent tubercle bacilli which was termed cord factor. Some of the biological properties of this extract suggested that it might be concerned with virulence. It was found later that the cord factor extracted with petroleum ether is not a pure substance, but a mixture of various compounds which differ biologically as well as chemically (19). The individual components have not yet been identified chemically.

One direct way to test the role of cord factor in virulence would be to compare the pathogenicity of tubercle bacilli before and after extraction with petroleum ether. This approach had to be abandoned because of the lack, at the present time, of suitable methods for resuspending extracted bacilli evenly without possible damage to them. Hence, it was not possible to prepare suspensions of equal numbers of viable bacteria for comparative infection experiments.

Because of this difficulty, efforts were made to dissociate experimentally the pathogenic properties of virulent bacilli from other manifestations of bacterial viability, for example the ability to give rise to colonies *in vitro* or to take up oxygen in respiration experiments. It seemed worth while to study the relationship between virulence and the production of cord factor in bacteria cultured under various conditions which may result in alterations of virulence. In a previous paper (5), it was shown that as bacterial cultures age, their virulence for mice drops markedly. This drop in virulence is accompanied by a loss in aging cultures of material extractable in petroleum ether (3).

In the experiments to be reported here, the relationship between virulence and the production of cord factor was studied in bacteria which had grown in

the presence of various amounts of tween 80 and also in organisms cultured in media containing small amounts of thiosemicarbazone compounds. Tween 80 was chosen because earlier observations had shown that bacteria grown in media with a high proportion of this detergent reduce methylene blue more readily than organisms cultured in the presence of small amounts of it (6). With bacilli grown in media with the usual small amounts of tween, the ability to decolorize methylene blue is characteristic of *non-virulent* variants of tubercle bacilli and of saprophytic mycobacteria (6–9). Large amounts of tween 80 were shown to cause virulent bacteria to act like avirulent organisms. It seems probable that this is due to a better and more rapid penetration of various substances into the bacterial cell, since tween has the ability to render virulent bacteria more sensitive to a variety of bacteriostatic and bactericidal agents (10–12). It was hoped, therefore, that the use of various concentrations of tween 80 would permit us to establish a quantitative relationship among surface permeability, the amount of cord factor extractable from the bacterial surface, and the virulence of the bacilli.

A comparison of the effects of therapeutically used thiosemicarbazone on bacterial virulence and on the production of cord factor was stimulated by recent observations on the effects of these compounds *in vitro* on bacterial morphology. Like tween 80 (1), thiosemicarbazones interfere with the formation of "cords" in cultures of virulent tubercle bacilli, thus bringing the morphologic appearance of these organisms close to that of non-virulent mycobacteria (13). Hence an attempt has been made in this case, too, to ascertain whether there is parallelism among colony morphology, bacterial virulence, and production of cord factor.

Since the course of an experimental infection is partly determined by the number of bacilli injected, it is essential that equal numbers of viable bacteria be compared whenever inferences are to be drawn on the virulence of a strain. Thus, one part of the present paper describes experiments in which the viability of bacteria grown under different circumstances was compared; a second part deals with comparative studies on the virulence of these organisms; and a third with the amounts and properties of the cord factor produced by these cultures under various conditions.

Materials and Methods

Bacteria.—For all the experiments, the H37Rv strain of the tubercle bacillus was used. Unless otherwise stated, the bacteria were propagated in 125 ml. Erlenmeyer flasks containing 30 ml. of tween-albumin liquid medium (14) with 0.05 per cent "certified tween 80" (Hill Top Laboratories, Cincinnati). The flasks were inoculated with 0.3 ml. of a 1 week old culture and incubated at 37.5°C. Many of the crucial experiments were duplicated with the bovine strain Vallée, but since the results were identical they are omitted from the following protocols. A modified Lockemann medium (15) was used for surface cultures.

Animals.—Male albino mice of the CF1 strain (Carworth Farms, New City, New York),

weighing between 15 and 20 gm., were used for infection experiments. The animals were infected intravenously with 0.1 ml. of bacterial suspensions. The toxicity of cord factor was tested on male C57 black mice received from Jackson Memorial Laboratories, Bar Harbor, Maine. These mice weighed about 15 gm.

Plate Counts.—The number of viable bacteria was determined according to the method of Fenner *et al.* (16, 17). Results were read after 3 weeks' incubation.

Turbidity Measurements.—Bacterial suspensions were standardized in a Coleman Junior spectrophotometer at a wave length of 550 mμ.

Methylene Blue Test.—The technique for this test has been briefly described in an earlier paper (6). Thunberg tubes of standard size contained 0.5 ml. bacterial suspension in a 1 per cent dextrose solution in M/15 phosphate buffer of pH 7.0 and 0.5 ml. of a 1:7000 dilution of methylene blue in water. The bacterial suspensions were washed in water and adjusted photometrically to a density indicating the presence of about 2 mg. bacteria (dry weight) per tube.

Respiration Experiments.—Bacteria grown in submerged cultures were washed and resuspended in culture medium, with tween added as indicated, and the suspensions photometrically adjusted to equal density. Surface grown bacilli were harvested after 2 weeks' growth, washed on the filter, and suspended as indicated after being gently ground in a mortar. The oxygen consumption was measured in a Warburg apparatus with air in the gas phase of the vessels.

Extraction of Cord Factor from Culture Filtrates.—5-liter flasks with 700 ml. of tween-albumin medium were inoculated with 5 ml. of a 1 week culture and incubated for 8 to 10 days. The bacilli were then separated from the medium by centrifugation and filtration through Seitz filters and the culture filtrates mixed with 2.5 volumes of methanol and 0.25 volumes of ether. After standing at −15°C. overnight, a heavy precipitate had formed which was collected by centrifugation in the cold. It was washed with methanol until it was free of tween 80,—which usually required three washings. The washed precipitate was then extracted for 4 hours with petroleum ether in a Soxhlet apparatus. After evaporation of the solvent, a yellowish, oily residue remained which was dissolved in ether and precipitated from methanol as a white, waxy material with a melting point of 35–36°C. The total yield from 700 ml. of culture filtrate was between 10 and 15 mg.

Para-formylacetanilide thiosemicarbazone (TBI)[1].—This compound was added to tween-albumin or Lockemann media in amounts of 0.2 to 0.5 μg. per ml. Under the culturing conditions used, between 15 and 25 μg./ml. was required for complete growth inhibition. In concentrations of less than 3.0 μg./ml., the growth rate of the cultures was not visibly affected.

Assay of Cord Factor Activity.—The most characteristic activity of cord factor is a delayed type of toxicity which can be assayed quantitatively in mice.

The material to be tested for toxicity was dissolved in light paraffin oil (bayol F). Before use, the clear solutions were heated to 37°C. and mice were then injected intraperitoneally with 0.1 ml. of 0.1 per cent solutions, thus receiving 0.1 mg. of the material to be tested at each injection. This amount represented the highest single dose given. Arbitrarily, preparations were considered inactive if repeated injections were without effect at this dosage. Control groups were given corresponding amounts of paraffin oil alone. Various vegetable oils (olive, sesame, poppy seed) or propylene glycol can be used instead of mineral oil, but bayol F proved to be the most convenient solvent.

[1] Samples of this compound were kindly supplied by the Monsanto Chemical Co., St. Louis.

Varying degrees of susceptibility to the effects of cord factor injections had been found in different strains of mice (3). Therefore, all tests were carried out with the same C57 Black strain received from the Jackson Memorial Laboratories, which was the most satisfactory. The animals were used within a few days after they arrived at our laboratory. They were housed in solid wall metal cages in groups of five and fed a standard pellet diet (Rockland mouse diet, Rockland Farms, New City, New York) and water *ad libitum*. The present paper reports experiments which extended over a period of more than 2 years. No intercurrent infections were observed in these mice during this time.

The assay was performed as follows:—Groups of at least five mice were injected with 0.1 cc. of a test dilution and the animals weighed daily in groups. Active preparations caused a loss of weight which became perceptible within a few hours after the injection. Depending on the preparation and the dose, the mice kept losing weight for several days. The activity of the animals was greatly reduced, their fur appeared wet and they lost as much as 20 per cent in weight. Usually after 2 to 3 days they began to regain it and reached their original weight after 4 to 7 days. A second injection was then given. If no loss of weight occurred, the mice were injected at 3 to 4 day intervals later. A test preparation was considered inactive when four injections of 0.1 mg. each did not cause any loss of weight. Moderately active preparations caused weight loss, but no death. Two to three injections of active preparations killed mice 1 to 2 weeks after the first injection. If three or less injections of one dilution killed at least three out of five mice within 15 days, the next smaller dose, usually a twofold dilution was tested until the threshold dose was reached.

At autopsy, the most conspicuous change in mice that had died from the injections was a massive hemorrhage in the lungs. The pulmonary volume was three to four times that of normal mice. In most cases, the lungs appeared uniformly dark red, whereas in some instances there were a few scattered areas of apparently normal, pink lung tissue left. Some mice had peritoneal exudates with fibrous adhesions, but there seemed to be no difference in this respect between animals injected with active preparations and control mice which were sacrificed after they had received several injections of bayol F. Histologically, the lung tissue of the experimental animals retained its structure, but the interalveolar tissue, as well as the vast majority of the alveoli, was packed with red blood cells and edema fluid. Capillaries and larger blood vessels were distended and filled with blood. The bronchi appeared free from cells and there were no signs of inflammation, except for some macrophages surrounding the capillaries. The hemorrhages appeared fresh. There were only few instances of organization. The congestion involved the entire lung tissue with no apparent focal distribution.

In rare instances, one mouse of a group in which the others died with the described symptoms showed a different picture: The hemorrhage was restricted to a few scattered areas, but the lungs were covered with grayish spots just barely visible to the naked eye. Histologically, these mice showed signs of lipid pneumonia. The pulmonary tissue was consolidated in many areas. The capillaries were dilated and the alveolar spaces contained a large number of vacuolated macrophages. A very few scattered oil droplets were present in these areas.

The Influence of Tween 80 and TBI on the Viability of Tubercle Bacilli

(*a*) *Growth Rate.*—Tween 80 was added to the standard medium to cover a range of final concentrations from 0.05 to 4.76 per cent. After 2, 4, and 8 days of incubation, the turbidity of these cultures and their number of viable units which formed visible colonies on solid media were determined (Table I). It can be seen that even very high amounts of tween 80 did not conspicuously affect the growth rate of tubercle bacilli. This suggests that the standard con-

centration of 0.5 per cent bovine albumin (fraction V) was sufficient to neutralize possible traces of free oleic acid, which would impair the bacterial growth (18) and indicates an adequate degree of purity of the preparation of tween 80 which was used. A later experiment in which five times the standard proportion of albumin was added to the medium yielded identical results (Table IV).

TABLE I

Number of Viable Organisms in Cultures Grown in Different Concentrations of Tween

Tween concentration	No. of colonies per ml. × 10^6			Light transmitted		
	Age of the cultures, *days*					
	2	4	8	2	4	8
per cent				*per cent*	*per cent*	*per cent*
0.05	0.32	2.01	9.81	97	94	78
0.15	0.21	2.51	8.64	97	93	79
0.45	0.30	2.24	5.93	97	93	81
1.35	0.35	2.42	5.31	97	95	81
2.25	0.38	2.81	5.31	97	94	81
3.17	0.37	1.97	5.13	97	94	83
4.76	0.36	1.91	5.08	97	95	82

TABLE II

*Number of Colonies grown in the Presence of TBI from Bacterial Suspensions Adjusted to Equal Optical Density**

Concentration of TBI in which the bacteria were grown	No. of colonies per ml. × 10^6
μg./ml.	
1.0	27.6
0.5	22.8
0.25	26.1
0.12	31.3
0.0	30.1

* The suspensions were prepared after the cultures had grown for 9 days in the presence of the amounts of TBI indicated in the table. Light transmission of the adjusted suspensions: 85 per cent.

The addition of 0.2 to 0.5 μg./ml. TBI to the medium was without visible influence on the growth rate and the number of viable bacterial units formed. When the drug concentrations were raised to 1.0 μg./ml., the rate of multiplication was somewhat slower than in the controls, but bacterial suspensions adjusted to equal density gave rise to an identical number of colonies when plated on solid media free from TBI (Table II).

(*b*) *Respiration Experiments.*—Another way of obtaining information about

the viability of a culture is to measure the rate of oxygen uptake of bacteria in a normal culture fluid.

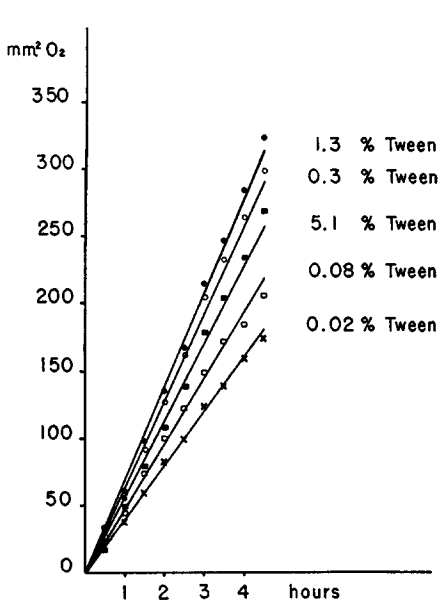

FIG. 1

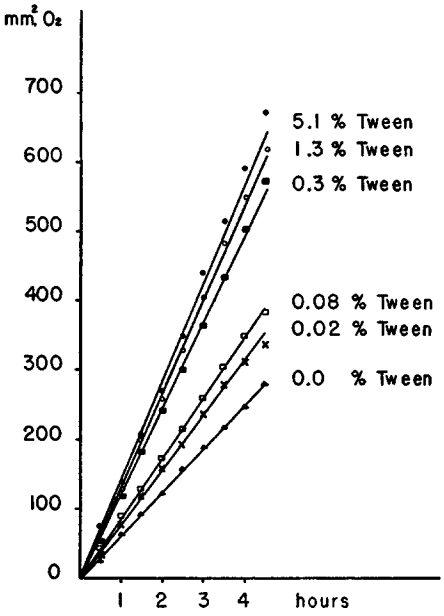

FIG. 2

FIG. 1. Oxygen uptake by tubercle bacilli grown in the presence of concentrations of tween 80 ranging from 0.02 per cent to 5.1 per cent. Each vessel containing approximately 1 mg. (dry weight) bacilli which were concentrated from 6 day old cultures after washing in saline, and resuspended in culture medium containing 0.05 per cent tween.

FIG. 2. Oxygen uptake by tubercle bacilli from an 11 day old surface culture. The ground bacilli were washed in saline and suspended in culture medium containing various concentrations of tween 80, ranging from 0.02 per cent to 5.1 per cent. Bacterial dry weight per vessel: approximately 9 mg.

This value was determined both with cultures grown in the presence of tween, and with bacteria cultured in the absence of tween and to which tween was added only in the Warburg vessel. For the first type of experiments, all cultures were washed in fresh medium containing 0.05 per cent tween and their oxygen uptake measured in this same type of medium. For the second type, surface-grown bacteria were ground in a mortar, washed, and suspended in basic medium containing albumin and varying amounts of tween 80. Figs. 1 and 2 represent typical experiments of the two varieties.

It can be seen that tween concentrations of up to 5.1 per cent did not reduce the oxygen uptake of the cells. Experiments with bacteria grown in the presence of 0.5 μg./ml. TBI, or with organisms to which TBI was added later, gave analogous results in that they failed to show an influence of these relatively small amounts of drug on the oxygen uptake of the bacilli (Table III).

The Effect of Tween 80 and TBI on the Virulence of Tubercle Bacilli

Groups of mice were infected intravenously with equal numbers of viable tubercle bacilli grown in the presence of various amounts of tween 80 and TBI. The data and outcome of these experiments are compiled in Tables IV and V. It is obvious from these tables that tween 80 or TBI, added to the culture media in proportions which did not affect the bacterial growth rate, had a considerable influence on the virulence of the organisms, as indicated by the survival times of the infected mice.

TABLE III

The Influence of TBI on the Oxygen Uptake of Tubercle Bacilli

Bacteria	Oxygen uptake, (*c.mm.*)* after			
	60 min.	120 min.	180 min.	240 min.
Grown on surface with 0.5 μg. TBI in the medium.	124	267	401	596
200 μg./ml. TBI added in the Warburg vessel (bacteria grown in Tween-albumin medium without TBI)‡	146	307	476	658
100 μg./ml. TBI added in the Warburg vessel (bacteria grown in Tween-albumin medium without TBI)‡	134	278	435	602
Control I§	150	315	496	695
Control II‖	69	128	189	261

* Average values of 3 vessels. Dry weight bacteria per vessel: 12.3 mg.

‡ The bacteria to which the drug was added were suspended in tween-albumin medium.

§ Oxygen uptake of bacteria in the same medium, but in the absence of drug.

‖ Oxygen uptake of bacteria in the same basic medium, but in the absence of TBI and tween 80.

In the case of tween 80, it is noteworthy that the loss in virulence was not directly proportional to the rise in the amounts of the detergent present in the medium. In the first experiment, a fiftyfold increase from 0.02 to 1.0 per cent tween resulted in a small but perceptible prolongation of the survival time of mice from an average of 23.8 to 28.7 days. In contrast, the next twofold increase from 1.0 to 2.0 per cent had a very pronounced effect with an increase in the average survival time from 28.7 to 76.3 days. The fact that the bacilli used for the second experiment were grown in the presence of 2.5 per cent albumin did not affect the results.

All infected animals (with the exceptions mentioned in the tables) died of tuberculosis. The autopsy findings were consistent with our previous experience (5): animals succumbing after a relatively short time died with evidence of an acute tuberculous disease, *i.e.* they had heart lesions with comparatively little involvement of the lungs, and disseminated inflammatory areas rich in tubercle

TABLE IV

Relationship between Virulence and Concentration of Tween in the Culture Medium

Experiment No.	Tween concentration in which the bacilli were grown	Age of culture	Bacterial count (colonies per ml. × 10^6)	No. of mice infected	Survival time*
	per cent	*days*			*days*
I‡	0.02	5	9.39	10	23.8 ± 1.5
	0.1		9.50	10	27.9 ± 3.9
	1.0		11.49	10	28.7 ± 1.5
	2.0		12.32	10	76.3 ± 13.2
II‡	0.05	6	8.7	20	17.1 ± 0.45
	0.175		6.09	20	20.5 ± 0.49
	0.612		9.09	19	24.2 ± 0.87
	2.14		10.9	20	98.25 ± 11.6§
III‖	0.175	5	11.75	10	22.2 ± 3.5
	0.612		10.93	10	24.8 ± 4.9
	2.14		9.98	10	69.0 ± 13.1¶

* With this method of infection, mice died of tuberculosis within a narrow range of time. The data listed in this table and Table V are characteristic, although some of the experimental groups are small. Of 218 mice infected during the past months in the same manner for other experiments than the ones reported here, the average survival time was 19.6 ± 0.26 days with cultures containing 0.05 per cent tween.

‡ Final concentration of albumin in these experiments: 0.5 per cent.

§ The last survivor of this group of mice was sacrificed 174 days after infection. It had conspicuous pulmonary lesions.

‖ Final concentration of albumin in this experiment: 2.5 per cent.

¶ Four of these mice were sacrificed after 114 days. In calculating the average survival time, these animals were noted as surviving for 114 days.

TABLE V

The Relationship between Virulence and the Amounts of TBI in the Culture Medium

Amount of TBI in the culture medium	Bacterial count (colonies per ml. × 10^6)*	No. of mice per group	Survival time
μg./ml.			*days*‡
0.5	37.2	17	50, 61, 80, 108, 122 (12)
0.1	32.9	17	19, 22, 22, 22, 23, 25, 26, 27, 39, 86, 98 (6)
0.02	29.1	7	22, 22, 23, 23, 24, 35, 52 (0)
—	31.2	17	7, 17, 20, 20, 20, 20, 21, 21, 21, 21, 23, 23, 24, 24, 24, 27, 108 (0)

* The mice were infected with 0.1 ml. of these suspensions.

‡ The numerals indicate the individual survival times. The figures in parentheses indicate the number of mice sacrificed after 138 days.

bacilli in most major organs; mice surviving for a longer period of time showed an increased total lung volume with extensive, confluent pulmonary lesions and only scattered foci in other organs.

The bacterial suspensions used for infection were photometrically adjusted to equal optical density. The slight variations in the number of bacilli, as determined by plate counts, are shown in Tables IV and V. They lie within the limits of error of the method and are of an order which cannot be detected by measuring the light transmission of a suspension. Although variations in the number of tubercle bacilli injected are one of the factors determining the survival time of mice, the data published by McKee *et al.* (4) showed that in a certain range of dosage a reduction of the infective dose by as much as 93 per cent merely doubled the mean survival time of the infected mice from 13.5 to 27 days. Table VI reports a similar experiment covering a somewhat smaller

TABLE VI

Relationship between Dose Administered and Survival Time of Mice Infected with Tubercle Bacilli

Infective dose	Light transmission (per cent at 550 mμ)	No. of mice per group	Average survival time
ml.			*days*
0.1	73	10	19.7 ± 0.75
0.075	80	10	19.9 ± 0.71
0.05	87	10	21.0 ± 0.47
0.025	93	10	21.8 ± 0.57

range of dilutions. It shows only a very slight prolongation of the survival time in a group of mice infected with one-fourth of the original infective dose. While a 1:4 dilution was conspicuously different optically from the original bacterial suspension, the variation between the average survival time of the two groups was minimal; hence, differences such as those reported in Tables IV and V cannot be attributed to minor irregularities in the adjustment of bacterial suspensions.

The Methylene Blue Test with Bacteria Grown in the Presence of Tween 80 and TBI

In this test, suspensions of saprophytic mycobacteria or of avirulent mutants of virulent strains of tubercle bacilli decolorize methylene blue within a few minutes whereas virulent bacteria grown in any one of the commonly used culture media do not reduce the dye. It has been suggested that this difference in behavior might be due to a different surface permeability of virulent and avirulent strains and be related to the distinctive growth patterns of the two varieties (6). Since high concentrations of tween 80 interfere with cord formation

in colonies of virulent tubercle bacilli (1), and since small amounts of TBI have a similar effect (13), the methylene blue test was performed on virulent tubercle bacilli cultured in the presence of various amounts of tween 80 and TBI. Typical experiments are presented in Tables VII and VIII. They show that

TABLE VII

The Reduction of Methylene Blue in Bacterial Suspensions from Media with Different Concentrations of Tween

Concentration of tween in the medium in which the bacteria were grown	Dry weight of bacteria per Thunberg tube	Decoloration of methylene blue after 30 min.
per cent	*mg.*	
0.02	2.0 ± 0.3	0
0.05		0
0.175		ic
0.612		c
2.14		c

The degree of decoloration is expressed by the following symbols:
0: no decoloration.
ic: incomplete decoloration.
c: complete decoloration.

TABLE VIII

The Reduction of Methylene Blue in Bacterial Suspensions from Media Containing Various Amounts of TBI

Concentration of TBI in the medium in which the bacteria were grown	Dry weight of bacteria per Thunberg tube	Decoloration of methylene blue after 30 min.*
μg./ml.	*mg.*	
1.0	2.0 ± 0.3	c
0.5		c
0.25		ic
0.125		0
0.0		0

* Symbols same as in Table VII.

under the standardized conditions of the test both TBI and tween 80 at a certain range of concentrations render the bacilli methylene blue–decolorizing.

The Effect of Tween 80 and TBI on the Formation of Cord Factor

If cord factor is related to the virulence of the bacillus, the changes in pathogenicity induced by tween 80 and by TBI should be associated with different yields of cord factor obtained from such bacteria. Surface cultures grown in the presence of 0.5 μg./ml. of TBI were harvested at the same time as control cultures grown on drug-free media and extracted in the usual way with pe-

troleum ether (3). The waxy fractions obtained were dissolved in light paraffin oil and injected into mice.

With tween 80 in the medium it was not possible to grow bacilli at the surface and the amounts which our laboratory facilities permitted us to grow in submerged cultures were too small to yield usable amounts of material extractable with petroleum ether. We tried, therefore, to obtain cord factor from filtrates of tween cultures. Previous observations had shown that stable suspensions of cord factor could be prepared in 2 per cent aqueous tween solutions. This fact suggested that bacteria grown in the presence of such large amounts of tween might not accumulate the cord factor they produced on their surface but rather yield it into the surrounding medium. Efforts were, therefore, made

TABLE IX

The Toxicity of Various Preparations of Cord Factor

Preparation	No. of mice per group.	No. of injections	Days from first injection to death	No. of survivors after 21 days
CF from surface culture (Control)	10	2–3	6, 7, 7, 9, 11, 12, 12, 14, 15, 17	0
CF from surface culture with 0.5 μg. TBI	10	4	2	9
CF from culture filtrate with 2 per cent tween	10	2–4	5, 8, 8, 8, 9, 11, 11, 13, 15, 15	0
Petroleum ether extract from culture filtrates with 0.05 per cent tween	10	4	0	10

to recover it from culture filtrates. Using the method described above, a waxy material was obtained which was dissolved in light paraffin oil and injected into mice. The results of several typical tests are shown in Table IX. It is obvious from these experiments that the material obtained from bacteria grown in the presence of TBI did not show the toxicity which is characteristic of the extracts from normal surface cultures of the same bacterial strain. The mice did not lose weight, nor did they die from the injections. On the contrary, the material extracted from the culture filtrates was active. All mice showed the characteristic loss of body weight and died with extensive hemorrhages in the lungs.

Thus, under the experimental conditions, TBI did not inhibit the growth of cultures, but it appeared to prevent the tubercle bacillus from forming a material having the delayed toxicity of cord factor and, like it, soluble in petroleum ether. In the tween experiments, such a material could be recovered from the

culture filtrates provided the proportion of tween in the medium was high, but no such material was found when the bacilli had grown in the presence of the usual concentration of 0.05 per cent tween 80.

TABLE X

Effect of High Concentrations of Tween upon Tubercle Bacilli

Mice infected with tubercle bacilli from*	No. of mice in group	Individual survival time	Survivor ratio after 78 days
		days‡	
5 day old culture grown in 0.05 per cent tween....	10	25, 28, 28, 30, 31	5/10
5 day old culture grown in 0.05 per cent tween, kept in medium with 2.1 per cent tween for 3 hrs. at 4°C.....	10	25, 26, 28, 30, 31, 33	4/10
5 day old culture grown in 0.05 per cent tween, incubated in medium with 2.1 per cent tween for 3 hrs. at 37°C.....	10	29, 29, 31, 38, 39, 42	4/10
5 day old culture, grown in 2.1 per cent tween medium.....	10	37	9/10

* The bacterial suspensions were adjusted to an equal density (94 per cent light transmission) before injection.

‡ The numerals indicate the day after infection on which the mice died from tuberculosis. The experiment was discontinued after 78 days and all surviving mice sacrificed. At autopsy, all the sacrificed animals showed conspicuous signs of pulmonary tuberculosis.

TABLE XI

The Effect of Tween Injections in Infected Mice

Infection with tubercle bacilli from*	Injections of tween 80				No. of mice in group	Average survival time
	Route of injection	No. of injections	Amount of tween per injection	Time over which injections extended		
			mg.	*days*		*days*
5 day old culture	—	—	—	—	10	18.6 ± 0.52
"	Intravenous	4	1.0	12	10	19.5 ± 0.47
4 day old culture	—	—	—	—	9	21.0 ± 0.7
"	Intraperitoneal	9	50.0	14	9	20.0 ± 0.96
"	"	9	25.0	14	10	21.7 ± 1.24
"	"	9	12.5	14	9	19.3 ± 1.65

* Light transmission of suspension: 87 per cent. This corresponds to the normal turbidity of a 5 day culture in tween-albumin medium.

These findings are in good agreement with the greatly reduced pathogenicity of bacteria grown in the presence of TBI or of high amounts of tween 80, as recorded in Tables IV and V.

Various attempts were made to ascertain if exposing bacterial cultures to

high amounts of tween, or to small quantities of TBI, results in changes similar to those occurring when the bacillus multiplies in the presence of these agents. All these attempts failed. The findings showed that:—(*a*) the virulence of tubercle bacilli was not reduced after they were suspended under various conditions in media containing 2 per cent tween 80 or 0.5 μg./ml. TBI (Table X); (*b*) virulent tubercle bacilli were not rendered methylene blue-decolorizing by the same procedures; (*c*) the action of virulent tubercle bacilli was not reduced by injections of tween 80 into tuberculous mice (Table XI).

RECAPITULATION AND DISCUSSION

The experiments described in the present paper were designed to test the hypothesis that a surface constituent of virulent tubercle bacilli extractable with petroleum ether is concerned in the pathogenicity of the organisms. This material had been termed cord factor because it appeared to be present only in cord-forming strains of tubercle bacilli, and because of the likelihood that its presence at the bacterial surface was instrumental in holding the organisms in their characteristic parallel arrangement after each cellular division.

Attempts at chemical purification of cord factor revealed that the material extracted with petroleum ether is a mixture of several components (19). These compounds seem to be closely related chemically, but only one fraction shows the type of delayed toxicity which characterizes all crude extracts from young virulent cultures. It was surmised that this toxicity could not be without consequence during the infectious process. The present experiments show that toxicity is lacking in petroleum ether extracts from tubercle bacilli grown in the presence of small amounts of TBI; that no toxic material can be obtained from culture filtrates of tween-albumin media containing 0.05 per cent tween, but that it is present in comparable culture filtrates when the bacteria have grown in the presence of 2 per cent tween. The good agreement between these facts and the findings that bacteria grown in the presence of the same amounts of TBI or tween 80 were considerably less virulent than bacilli of the same strain grown under the usual conditions is suggestive of the role played by the cord factor during infection. Under the conditions in which the formation of cord factor was inhibited (TBI), or in which it was secreted into the surrounding medium (high concentration of tween 80), the bacilli, though viable, were less virulent.

Virulence, in these experiments, is defined only in terms of the time period elapsing before death. This is obviously a simple standard, but we do not know of any other available criterion having the same accuracy in murine tuberculosis. Variations in the host can be minimized by keeping animals of the same breed under standardized conditions and observed differences can be safely attributed to the action of the infecting organisms. Evidence has been presented

to show why we believe that differences in the survival times, as reported in the present paper, cannot be attributed to technical errors in the preparation of suspensions of equal numbers of viable cells. It appears that at a certain concentration of TBI or tween 80 in the culture medium, the virulence of the tubercle bacillus is affected, whereas its ability to multiply *in vitro* remains unchanged.

With tween 80, the oxygen uptake of the organisms is even higher than that of bacteria grown in ordinary media. The increased oxygen uptake of washed cultures grown in the presence of higher concentrations of tween may be due either to more tween still adhering to the cells and being metabolized, or to the fact that the long contact with high concentrations of tween has rendered the bacterial surface more permeable to the nutrients in the surrounding medium. This latter possibility may also account for the increased oxygen uptake encountered after the addition of tween to bacteria grown in the absence of it. Regardless of the mechanism, the experiments demonstrate an uninhibited respiratory activity in bacteria from 1 week old cultures grown in the presence of large amounts of tween. It may be recalled that oxygen uptake alone is not a true indicator of the viability of the cells (20), but when considered together with plate counts it serves as collateral evidence.

The loss of virulence effected by tween did not increase proportionately with the concentration of the detergent in the medium, but there was a critical threshold dose at which the change occurred. A fiftyfold increase of tween, from 0.05 to 1.0 per cent, caused only a slight prolongation of the survival time by about 20 per cent, whereas the next twofold increase induced an extension of the average survival time by more than 300 per cent. Since a bactericidal effect of tween at these concentrations was ruled out by the control experiments, the reduced virulence of the organisms is most likely due to an action affecting selectively some cellular properties or components which are more essential for the survival and multiplication of the bacillus *in vivo* than *in vitro*.

At the present time, there is no way of telling at what phase of the infectious process the differences among organisms grown under various circumstances are most likely to appear. The simplest assumption is that at an early stage, shortly after infection, a certain proportion of the injected bacteria are destroyed by the host's cellular defense mechanisms. A working hypothesis has been presented earlier (3) that cord factor acts as a cover protecting the bacteria from being digested by phagocytes. In the present experiments, some of the bacteria had less of this coating; its synthesis was either inhibited by TBI, or the high amounts of tween prevented it from accumulating at the bacterial surface. Fewer bacteria may have survived phagocytosis, the effect being comparable to an infection with fewer bacilli. *In vitro*, when no antibacterial agents or mechanisms keep the bacilli from multiplying freely, each viable unit is able to give rise to a colony, regardless of how well protected the or-

ganisms are against external influences. This would account for the discrepancy between the number of viable bacteria as determined by plate counts, and the reduced pathogenicity of these bacterial suspensions.

The therapeutic action of thiosemicarbazones might be at least partly explained as due to similar mechanisms. In contrast to streptomycin, these compounds have no sharp end-point on test *in vitro* for growth inhibition, and *in vivo* the blood levels reached are often too small to account for a therapeutic effect on the basis of their bacteriostatic action alone.

SUMMARY

Tubercle bacilli were grown in the presence of different concentrations of tween 80, ranging from 0.05 to 2.1 per cent. Equal numbers of viable bacteria from these cultures were compared in infection experiments in the mouse. The average survival time of the mice was used as a criterion for the virulence of the bacilli. High tween concentrations in the culture medium caused a reduction of the bacterial virulence. The reduction was slight in bacterial suspensions from cultures with tween 80 ranging from 0.05 to 1.0 per cent, but considerable in cultures with 2.1 per cent tween.

Bacteria grown in the presence of 2.1 per cent tween gave rise to the same number of colonies, *in vitro*, as bacteria grown in ordinary media. Their oxygen uptake was increased as compared with that of bacilli grown in media containing less tween.

Virulent bacteria grown in the presence of high amounts of tween 80 decolorized methylene blue in a test in which organisms from the same virulent strain but cultured without tween, or with only small proportions of the detergent in the medium, did not reduce the dye. A positive methylene blue test is typical of non-virulent tubercle bacilli and of saprophytic mycobacteria.

Essentially the same changes occurred when virulent tubercle bacilli were grown in the presence of 0.5 μg./ml. of para-formacetanilide thiosemicarbazone (TBI). This small amount of the substance was not sufficient to prevent the growth of bacteria, or to reduce the number of viable cells in a culture, but it reduced the virulence of the bacteria considerably and rendered them capable of decolorizing methylene blue.

Cord factor, a lipid constituent of virulent bacteria which is toxic for mice, was shown to be present in filtrates from cultures of virulent bacteria when the media contained 2 per cent tween 80, but no such material could be recovered from culture filtrates containing the usual 0.05 per cent tween. On the other hand, no toxic material could be extracted from bacteria grown in the presence of 0.5 μg./ml. TBI.

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