MULTIPLICATION AND SURVIVAL OF TUBERCLE BACILLI IN THE ORGANS OF MICE

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It has been known for more than half a century that virulent tubercle bacilli can undergo hereditary modifications lessening their pathogenicity. Quantitatively, the decrease in virulence can be measured in terms of the severity and duration of the lesions produced in experimental animals by the injection of known doses of living bacilli. The results obtained by this technique suggest that each of the variant forms of tubercle bacilli is endowed with a characteristic type of behavior *in vivo*.

Many attempts—not to be reviewed here—have been made to correlate the levels of virulence with the morphological and biochemical characteristics of the culture variants, but such studies have not yet thrown any light on the properties which determine the degree to which tubercle bacilli are capable of causing disease. It is still unknown whether the difference in virulence of the culture variants depends upon the ability of the bacterial cells to multiply, to survive, or to exert toxic effects *in vivo*. The experiments to be described in the present paper were designed to determine the comparative rate and extent of multiplication *in vivo* of strains of tubercle bacilli known to differ in their pathogenicity for a variety of experimental animals. The level of virulence of these strains had been evaluated previously in this and other laboratories in terms of the extent of lesions and of the mortality caused by the injection of known amounts of culture into rabbits, guinea pigs, and mice. It will be shown that for each strain, the degree of virulence revealed by these techniques bears a close relation to the numbers of living bacilli found to be present in the tissues of mice at various intervals of time after inoculation under defined conditions with known amounts of culture.

Materials and Methods

Cultures.—The following strains of tubercle bacilli were used:—

H37Rv (human type).—Obtained through the courtesy of Mr. W. Steenken, Jr., from the Standard Culture Depot, maintained by the National Tuberculosis Association at the Trudeau Laboratory (Saranac, New York). The history of this strain is described in reference 1.

Amerzanga (human type).—Obtained through the courtesy of Doctor J. Aronson, Henry Phipps Institute, Philadelphia.

Ravenel (bovine type).—Obtained through the courtesy of Doctor J. Freund, New York City Public Health Research Institute.

MV (Vallée) (bovine type).—Originally obtained from the Pasteur Institute, Paris; this strain has been repeatedly passed through mice and reisolated from mouse tissue in our laboratory.

R1Rv (human type).—Obtained through the courtesy of Mr. W. Steenken, Jr., from the Standard Culture Depot, maintained by the National Tuberculosis Association at the Trudeau Laboratory (Saranac, New York). The history of this strain is described in reference 2.

BCG-P (bovine type)—A substrain of BCG obtained through the courtesy of Doctor J. Aronson, Henry Phipps Institute, Philadelphia.

BCG-T (bovine type).—A substrain of BCG obtained through the courtesy of Doctor S. Rosenthal, Tice Laboratories, Chicago.

H37Ra (human type).—Obtained through the courtesy of Mr. W. Steenken, Jr., from the Standard Culture Depot, maintained by the National Tuberculosis Association at the Trudeau Laboratory (Saranac, New York). The history of this strain is presented in reference 3.

Strains H37Rv, Amerzanga, Ravenel, and MV are capable of causing progressive disease and death in normal guinea pigs and mice. Strains R1Rv, BCG-P, and BCG-T can produce an abortive disease with self-limited lesions in guinea pigs and mice. The comparative pathogenicity of these strains for mice has been recently described in papers from this laboratory (4–6). For a complete and critical review of the literature on mouse tuberculosis see reference 7.

In order to preserve stocks of strains of tubercle bacilli in their characteristic state of virulence, the spleens of mice infected with the bacilli were desiccated and stored in the following manner: A volume of 0.2 ml. of culture was injected into mice by the intravenous route. The animals were sacrificed 2 weeks later, and their spleens removed aseptically. The infected spleen tissue was minced, distributed in small precipitin tubes, and desiccated in the cold, *in vacuo*, over P_2O_5. The tubes were then sealed with pyseal and stored at room temperature.

Cultures were recovered from these samples by adding liquid tween-albumin medium to the desiccated spleen tissue which contained large numbers of living tubercle bacilli. Growth occurred after 1 week's incubation at 37.5°C.

In the course of experimentation, all strains were maintained by weekly passages in tween-albumin medium (8). The cultures were 7 to 10 days old at the time of use.

Animals.—All experiments were carried out with albino mice (so called Rockefeller Swiss strain) raised at The Rockefeller Institute for Medical Research. They were received in the laboratory 1 or 2 days after weaning, at approximately 3 weeks of age. They were fed pellets with water *ad lib.*, and were housed either in metal cages, or in glass jars. By preference they were infected (or vaccinated) within 1 week, or 2, after being received in the laboratory.

Infection was carried out by injecting 0.03 ml. of the proper dilution of culture by the intracerebral route, or 0.2 ml. by the subcutaneous, intraperitoneal, or intravenous route. The dilutions were made directly from 7 to 10 day old cultures in tween-albumin medium into a diluent consisting of 0.1 per cent bovine albumin (bovine plasma fraction V). When mice were vaccinated[1] with attenuated or avirulent cultures before being given the virulent challenge infection, bovine albumin was used as diluent for one of the injections (usually for injecting the culture used as vaccine) and albumin from another species for the other injec-

[1] Vaccination experiments are described in the two following papers of this issue.

tion. This avoided the danger of anaphylactic shock occasionally encountered when solutions of the same protein used in the culture medium and as diluent were injected at a few weeks' interval. In general, the preparation used in lieu of purified human albumin was the soluble fraction of human serum heated at acid pH described in earlier publications (9).

Preparation of Tissue Emulsions.—Mice were killed with chloroform and their organs were removed aseptically; the blood was obtained by heart puncture.

The organs were ground in the presence of 2 per cent bovine serum albumin; this protein served the purpose of protecting the bacilli from the toxic effects of substances released by grinding, or by autolysis, of the tissues. For the same reason, all further dilutions were made in 0.1 per cent bovine serum albumin (or human albumin).

During the early phase of the experiments, the organs were ground with a mortar and pestle in the presence of 90 mesh alundum (crystalline Al_2O_3). More recently, a "teflon" homogenizer was used.

The teflon homogenizer (Figs. 1 and 2) is a modification of the lucite unit described by Brendler (10). Teflon is the du Pont trademark for a plastic consisting of a tetrafluoroethylene resin produced by polymerization of the gaseous compound. It is remarkably inert to organic solvents, strong acids, or alkalis, and can withstand high temperatures. These properties greatly facilitate its sterilization and for this reason give it superiority over lucite and other plastics in bacteriological experiments.

Two inch lengths of teflon rods were machined to fit the inside of ignition tubes, and a stainless steel shaft was inserted into the teflon.[2] The free end of the shaft was fashioned to fit a specially designed chuck which allows easy introduction and locking of the shaft. As the inside of the ignition tubes is of standard contour, the teflon rods and ignition tubes are interchangeable.

In practice, the rods and tubes were autoclaved separately, and assembled after the organ to be ground and 5 ml. of 2 per cent serum albumin had been deposited in the tube. During operation, the teflon rod was caused to revolve at 1740 R.P.M. by a motor operated by a foot control. Homogenization of the tissue required approximately 30 seconds, during which time the tube containing the organ and albumin solution was pushed up and pulled down the revolving teflon rod approximately ten times. Since no abrasive was used, homogenization was accomplished by shearing action alone.

The suspension of homogenized tissue was found to consist of ruptured tissue cells and intact nuclei, among which tubercle bacilli could be seen to occur singly or in small aggregates.

Enumeration of Living Tubercle Bacilli.—The numbers of tubercle bacilli present in the cultures used for infection or in the emulsions of organs recovered from infected mice were determined by plating appropriate dilutions on oleic acid–albumin agar (8). However, in the case of experiments in which the BCG-T culture was used, the solid medium was prepared with albumin solution instead of the oleic acid–albumin complex. This was necessary because it has been our general experience that the growth of the BCG-T culture is often somewhat inhibited by the concentration of oleic acid used in the oleic acid–albumin complex. In comparative tests carried out with both media, the numbers of colonies obtained from all other strains were independent of the medium, but the colonial size was larger on the oleic acid–albumin agar.

Cultures as well as tissue emulsions were diluted in 0.1 per cent albumin (in distilled water), and one drop of the proper dilution was deposited on the dry surface of an agar plate. The solid medium was incubated for 24 hours at 37.5°C. before use in order to check

[2] Designed and made by Mr. Josef Blum (Instrument Shop, The Rockefeller Institute for Medical Research). Now produced commercially by Scientific Glass Apparatus Company, Bloomfield, New Jersey.

sterility and to insure a surface of correct moisture content. The drops of the suspension to be assayed were delivered from micropipettes or tuberculin syringes, or more commonly from Pasteur capillary pipettes. Each drop contained approximately 0.025 ml. of fluid. The plates were incubated at 37.5°C. in plastic bags (to prevent evaporation). The colonies of tubercle bacilli were counted after 14 days' incubation at 37.5°C.

In practice, three dilutions of organ emulsions were plated: the original suspension made up of the whole organ homogenized in 5 ml. of 2 per cent albumin solution, and 10-fold and 100-fold dilutions of this original suspension. All platings were done at least in duplicate. From the numbers of colonies obtained at the proper dilution, *i.e.* one giving preferably 5 to 50 colonies per drop, it was easy to calculate the total number of colonies yielded per each organ (or per milliliter of blood, or of culture). See Figs. 3 and 4.

Bacteriological Hood for the Manipulation of Infected Materials.—All procedures necessary for the infection and autopsy of mice, the grinding of tissues, and for diluting and plating the infected suspensions or virulent cultures, were carried out in hoods specially designed to protect workers against infection. The hoods are completely enclosed except for two armholes. They were kept under a slight negative pressure by an exhaust motor pulling air from the chamber through a filter. The interior of the hood was sterilized by ultraviolet irradiation before and at the completion of each experiment. Irradiation was discontinued during the experiment.

The transfers and dilutions of infected suspensions and of virulent cultures were made by means of tuberculin syringes with No. 22 gauge, 2 inch needles. No mouth pipettes were used, the plating being done with Pasteur capillary pipettes controlled by means of rubber bulbs. The Pasteur pipettes were drawn to deliver approximately 0.025 ml. per drop.

RESULTS

In the preliminary phase of this study, experiments were carried out to compare the effectiveness of different routes of injection of the bacilli into mice—subcutaneous, intraperitoneal, intracerebral, and intravenous. It was soon recognized that most of the bacilli injected subcutaneously or intraperitoneally were trapped at the site of injection and that the numbers of colonies recovered from other tissues were always erratic and often small, even when the infective dose was large. By contrast, minute infective doses introduced intravenously or intracerebrally were sufficient to initiate in the spleen, liver, and lungs, a bacillary multiplication that could be readily followed by bacteriological techniques. For reasons of convenience, the intracerebral route of injection was used in the early phase of the work. Later it was found that more reproducible results could be obtained by introducing the infective inoculum intravenously, and this route of injection has been used by preference during the past 2 years. The results obtained by intravenous infection have confirmed in all regards the conclusions derived from the early experiments in which the intracerebral route was used.

Distribution of Tubercle Bacilli in the Different Organs of Mice Following Intracerebral Injection.—

Mice were injected by the intracerebral route with 0.003 ml. of one of the following three virulent cultures: H37Rv, Amerzanga, and Ravenel. In the case of each culture, two animals

were sacrificed immediately (within 5 minutes) after injection, and the others 24, 72, 96, and 188 hours later. Blood samples obtained by cardiac puncture, and organ suspensions prepared as described under Materials and Methods were diluted in 0.1 per cent albumin. The dilutions were plated on oleic acid–albumin agar. The numbers of bacterial colonies per milliliter of blood or per whole organ of each mouse are recorded in Table I.

TABLE I

Multiplication of Virulent Tubercle Bacilli in the Organs of Mice Infected by the Intracerebral Route

Infection		Period after injection	No. ($\times 10^{-2}$) of colonies recovered from							
Strain	Dose		Blood		Brain		Lungs		Spleen	
	ml.	*hrs.*								
H37RV*	0.03×10^{-1}	0	110‡	0	450‡	240	3‡	0	0‡	0
		24	0	0	570	220	0	0	3	0
		72	0	0	900	330	0	0	135	11
		96	0	0	2,000	200	0	0	400	26
		188			8,800	4,000	3	1	500	200
Ravenel*	0.03×10^{-1}	0	440	230	2,500	1,700	14	2	18	9
		24	0	0	2,500	2,300	0	0	52	22
		72	0	0	12,600	2,000	4	1	1,600	330
		96	0	0	>20,000	5	7	0	1,000	10
		188	0	0	>20,000	>20,000	14	9	1,200	1,200
Amerzanga*	0.03×10^{-1}	0	290	0	190	190	2	0	0	0
		24	0	0	930	320	0	0	58	5
		72	0	0	5,500	3,700	0	0	150	105
		96	0	0	6,500	6,300	40	5	350	280
		188	0	0	>20,000	8,400	24	7	8,500	3,400

* The cultures used for infection contained the following numbers of viable organisms, single or small clumps, per milliliter of culture (as determined by plating on oleic acid–albumin agar).

H37Rv..... 2.0×10^7
Ravenel..... 15.6×10^7
Amerzanga..... 4.8×10^7

‡ The figures in the table, when multiplied by 100, give calculated numbers of colonies of tubercle bacilli that could be recovered on oleic acid–albumin agar per milliliter of blood or per whole organ. (Each figure corresponds to the average of two platings per mouse.)

The results presented in Table I show that large numbers of tubercle bacilli could occasionally be recovered from the blood stream within a few minutes following intracerebral inoculation. However, the bacilli never could be cultivated from the blood after that time despite the fact that they had multiplied in other organs. Thus, it is evident that intracerebral inoculation had allowed extensive passage of bacilli into the general circulation, but that the

clearing mechanisms of the body removed them from the blood rapidly and thoroughly.

Large numbers of bacilli were of course present in the brain from the beginning, and they progressively increased in this organ throughout the period of observation. Only small numbers could be recovered from the spleen and the lungs during the first few days. Then they increased rapidly in the spleen but much more slowly in the lungs. The difference in behavior of the bacilli in these two latter organs has been consistently observed following intracerebral and intravenous injection in all similar experiments. It is only in the later phases of the infection that the numbers of bacilli in the lungs have been observed to increase significantly.

Effect of the Size of the Infective Dose on the Multiplication of Virulent Tubercle Bacilli in the Spleen and Lungs of Mice Injected Intracerebrally.—

Mice were infected by the intracerebral route with 0.003, 0.0003, or 0.00003 ml. of culture of the virulent strain Ravenel. Groups of 4 to 10 animals for each infective dose were sacrificed at intervals of time (up to 12 weeks) and the numbers of living bacilli present in the spleen or lungs were determined by plating dilutions of these organs on oleic acid–albumin agar. The results are presented in Table II.

In agreement with the results presented in Table I, far larger numbers of bacilli were recovered from the spleen than from the lungs during the first 2 weeks after infection. But in all cases the numbers of bacilli in the spleen stopped increasing or began to decrease 2 to 3 weeks after infection, and this has been a constant finding in all experiments. By contrast, the bacterial population in the lungs continued to increase for a much longer time, indeed until the death of the animal, provided the infective inoculum was large enough.

Effect of Virulence of the Strain on the Multiplication of the Bacilli in the Spleens of Mice Infected by the Intracerebral Route.—

Groups of mice were infected by the intracerebral route with 0.03 ml. of culture of either one of the three following strains of tubercle bacilli: the virulent strain H37Rv, its avirulent variant H37Ra, and the attenuated strain R1Rv. The strains all belonged to the human type, but as can be seen, differed greatly in virulence. The animals were sacrificed at different intervals of time after infection, and the numbers of living bacilli in their spleens were determined by the usual techniques (Table III).

The three cultures of human type used in this experiment were of the same age and of approximately the same optical density. Plated on oleic acid–albumin agar, they yielded approximately the same number of bacterial colonies, (3.2×10^8, 4.8×10^8, and 3.6×10^8 respectively per ml. of culture of H37Rv, R1Rv, and H37Ra). This quantitative similarity in culture population was reflected in the similar numbers of bacilli that were recovered from the spleens

24 hours after injection into mice. The qualitative differences among the three strains appeared clearly from the change with time in the numbers of living bacilli present in the tissue. In the case of the strain H37Ra, the numbers of living bacilli in the spleen decreased by two-thirds during the 1st week and continued to decrease thereafter. This culture has been shown in many

TABLE II

Effect of Size of Infective Dose on Multiplication of Virulent Tubercle Bacilli (Ravenel) Following Intracerebral Injection into Mice

Dose of culture* injected	Time after injection	No. ($\times 10^{-2}$) of colonies recovered from	
		Spleen	Lungs
ml.			
0.03×10^{-1}	1 day	48‡	0‡
	1 wk.	3,858	22
	2 wks.	2,008	2,318
	4 "	170	1,090
	6 "	180	4,780
	12 "	14	56,000
0.03×10^{-3}	1 day	116	2
	1 wk.	1,938	18
	2 wks.	636	16
	4 "	92	50
	6 "	118	370
	12 "	4	0
0.03×10^{-5}	1 day	2	0
	1 wk.	40	2
	2 wks.	678	14
	4 "	64	30
	6 "	32	18
	12 "	0	0

* The Ravenel culture used for infection contained 1.6×10^8 viable organisms, single or small clumps, per milliliter of culture (as determined by plating on oleic acid–albumin agar).

‡ The figures in the table, when multiplied by 100, give calculated numbers of colonies of tubercle bacilli that could be recovered on oleic acid–albumin agar per whole organ. (The figures given are averages for 4 to 10 animals—duplicate platings.)

earlier studies to be unable to produce disease or lesions in guinea pigs, rabbits, or mice (3–5, 7). In so far as can be judged, therefore, it has lost the ability to multiply *in vivo*.

With both strains H37Rv and R1Rv there was a marked increase in the numbers of bacilli in the spleen during the 1st week, but the numbers did not continue to increase much longer. In fact, the numbers decreased slightly during subsequent weeks, even in animals which died of infection with the viru-

lent culture H37Rv. Although extensive multiplication of the culture R1Rv was observed at first, and the numbers of surviving bacilli in the spleens remained high thereafter, the mice survived the infection and remained in apparent good health. These observations are in keeping with the fact that

TABLE III

Effect of Virulence of Tubercle Bacilli on Their Multiplication in Spleens of Mice Following Intracerebral Injection

Infection		Time after injection	No. ($\times 10^{-3}$) of colonies recovered from spleen
Culture	Dose		
	ml.		
Virulent (H37Rv)*	0.03	1 day	1,228‡
		1 wk.	16,990
		2 wks.	9,110
		5 "	5,810
		8 "	All mice dead from infection
Attenuated (R1Rv)*	0.03	1 day	1,436
		1 wk.	6,302
		2 wks.	726
		5 "	1,666
		8 "	1,798
Avirulent (H37Ra)*	0.03	1 day	2,288
		1 wk.	650
		2 wks.	260
		5 "	10
		8 "	0

* The cultures used for infection contained the following numbers of viable organisms, single or small clumps, per milliliter of culture (as determined by plating on oleic acid–albumin agar).

H37Rv..... 3.2×10^8
R1Rv..... 4.8×10^8
H37Ra..... 3.6×10^8

‡ The figures in the table, when multiplied by 100, give calculated numbers of colonies of tubercle bacilli that could be recovered on oleic acid–albumin agar per whole organ. (Each figure corresponds to the average for ten mice—duplicate platings.)

cultures of R1Rv injected into normal guinea pigs and mice give rise to fairly extensive lesions which remain self-limited and rarely lead to a fatal outcome (2, 5).

Fate of Avirulent Bacilli in Tissues.—

Mice were infected by the intracerebral route with the avirulent culture H37Ra; the number of living bacilli injected was approximately one-third that injected in the preceding

experiment. Groups of mice were sacrificed at several intervals of time and the bacterial population in the brain and spleen was determined by the usual techniques (Table IV).

In confirmation of the results presented in Table III it was found that the numbers of avirulent bacilli H37Ra in the spleen decreased progressively throughout the period of observation (Table IV). This occurred also in the brain, despite the trauma that had been caused by the injection. But even in the case of this completely avirulent strain, living bacilli persisted in the tissues for many weeks. It appears therefore that bacilli of the strain H37Ra fail to cause lesions not because they are susceptible to a bactericidal effect of

TABLE IV

Survival of Avirulent Human Tubercle Bacilli (H37Ra) in the Brain and Spleen of Mice Injected Intracerebrally

Time after intracerebral injection of 0.03 cc. culture H37Ra*	No. ($\times 10^{-2}$) of colonies recovered from	
	Brain	Spleen
1 day	18,600*	310
2 wks.	2,900	218
3 "	796	86
4 "	692	8
8 "	4	2

* The H37Ra culture used for injection contained 17.0×10^7 viable organisms, single or small clumps, per milliliter of culture (as determined by plating on oleic acid–albumin agar).

‡ The figures in the table, when multiplied by 100, give calculated numbers of colonies of tubercle bacilli that could be recovered on oleic acid–albumin agar per whole organ. (Each figure corresponds to the average for four mice—duplicate platings.)

the cellular or humoral defense mechanisms, but because they are incapable of multiplying *in vivo*.

Effect of the Route of Injection on the Multiplication of Bacilli in the Spleen and Lungs of Mice.—Although no systematic study has been made of the comparative distribution and fate of bacilli injected into mice by different routes, it was noticed in the course of preliminary observations that the numbers of bacilli recovered from the different organs were largest when the injection was intravenous. The intracerebral, intraperitoneal, and subcutaneous routes came next in order of effectiveness. Moreover, results by the intravenous technique were always the most reproducible. The following experiments are presented to illustrate these findings.

In one experiment 3×10^{-5} ml. of the culture R1Rv was injected into mice either by the intravenous route (0.3×10^{-4} ml.) or by the intracerebral route (0.03×10^{-3} ml.).

In another experiment two different doses of the culture BCG-P (0.3×10^{-3} ml. or 0.3×10^{-5} ml.) were injected into mice either intravenously or intraperitoneally.

The animals were sacrificed at different intervals of time after injection, and the numbers of living bacilli in their spleens and lungs were determined. The results for each individual mouse, for the 2 week and the 5 week period after infection, are presented in Table V.

It is clear that many more living bacilli (approximately tenfold) were present in the lungs of the animals infected with the strain R1Rv by the intra-

TABLE V

Effect of Route of Injection on Multiplication of Tubercle Bacilli in Mice

Injection		Route of injection	No. ($\times 10^{-2}$) of colonies recovered from spleens and lungs of mice sacrificed 2 or 5 wks. after injection of tubercle bacilli											
			2 wks.							5 wks.				
Strain	Dose		Spleen				Lungs			Spleen			Lungs		
	ml.													
R1Rv*	0.03×10^{-3}	Intracerebral	3,600‡	700	—	—	40‡	4	—	Not done			Not done		
R1Rv	0.3×10^{-4}	Intravenous	2,100	1,900	1,700	1,500	640	460	112	"	"		"	"	
BCG-P	0.3×10^{-3}	Intravenous	1,780	1,260	—	—	0	0	—	1,060	700	330	1,844	1,040	860
BCG-P	0.3×10^{-5}	Intravenous	—	—	—	—	0	0	0	85	44	—	76	5	—
BCG-P	0.3×10^{-3}	Intraperitoneal	190	100	—	—	0	0	0	5	3	—	11	1	0
BCG-P	0.3×10^{-5}	Intraperitoneal	5	0	0	—	0	0	0	0	0	0	0	0	0

—, the count was unavailable for technical reasons.

* The R1Rv culture used for infection contained 3.6×10^8 viable organisms, single or small clumps, per milliliter of culture (as determined by plating on oleic acid-albumin agar). No count was obtained for the original inoculum of BCG-P because of accident.

‡ The figures in the table, when multiplied by 100 give calculated numbers of colonies of tubercle bacilli that could be recovered on oleic acid-albumin agar per whole organ. (Each figure corresponds to the average of two platings per mouse.)

venous route than in those of mice receiving the same infective dose intracerebrally. The difference was even greater between the intravenous and intraperitoneal routes and this was true for the spleen as well. In fact, whereas injection of 0.3×10^{-3} ml. of BCG-P by the intraperitoneal route gave rise to only a limited and erratic infection, 0.3×10^{-5} ml. given by the intravenous route was sufficient to infect all animals.

Just as in the case of intracerebral injection, the numbers of bacilli in the spleen were much larger than in the lungs during the first 2 weeks following intravenous injection. As the infection progressed, however, the numbers in the lungs continued to increase.

TABLE VI

Effect of Virulence of Tubercle Bacilli on Their Multiplication in Mice Following Intravenous Injection of Small Doses of Culture

Infection		No. ($\times 10^{-2}$) of colonies recovered from mice sacrificed at several periods of time after intravenous injection of tubercle bacilli									
		Spleen					Lungs				
		Time in wks.					Time in wks.				
Strain*	Dose	1	2	3	5	7	1	2	3	5	7
	ml.										
MV, virulent	0.3×10^{-5}	480‡	6,400	3,000	1,300	710	0‡	410	36	800	5,200
		290	6,400	2,600	1,140	—	0	160	—	—	590
		90	6,200	—	920	—	0	46	—	—	—
	0.3×10^{-6}	26	940	1,700	300	550	0	0	0	96	2,910
		12	880	340	270	310	0	0	0	18	270
		—	—	—	190	—	0	0	0	10	—
	0.3×10^{-7}	0	60	100	2,700	180	0	0	0	340	1,710
		0	30	64	400	170	0	0	0	6	6
		—	—	46	270	—	0	0	0	—	—
BCG-P, attenuated	0.3×10^{-4}	18	88	460	310	60	0	4	10	62	28
		16	32	260	300	—	0	2	8	14	18
		—	—	220	280	—	0	—	2	—	—
	0.3×10^{-5}	2	4	48	250	28	0	0	2	2	24
		2	2	30	116	—	0	0	0	2	4
		0	2	30	70	—	0	0	0	2	—
	0.3×10^{-6}	0	0	24	34	24	0	0	0	0	0
		0	0	6	32	22	0	0	0	0	0
		0	0	4	24	20	0	0	0	0	0
BCG-T, attenuated	0.3×10^{-4}	116	—	280	40	26	24	0	2	2	0
		60	—	260	16	12	2	0	2	2	0
		—	—	88	—	8	—	0	0	0	0
	0.3×10^{-5}	2	2	26	30	8	0	0	2	0	0
		2	—	20	10	6	0	0	0	0	0
		0	—	8	—	0	0	0	—	0	0
	0.3×10^{-6}	0	—	2	10	4	0	—	0	0	0
		0	—	2	0	2	0	—	0	0	0
		0	—	2	0	0	0	—	0	0	0

—, the count was unavailable for technical reasons.

* The cultures used for infection contained the following numbers of viable organisms, single or small clumps, per milliliter of culture (as determined by plating on albumin agar).

MV..... 8.0×10^7

BCG-P..... 3.2×10^7

BCG-T..... 2.8×10^7

‡ The figures in the table, when multiplied by 100, give calculated numbers of colonies of tubercle bacilli that could be recovered on albumin agar per whole organ. (Each figure corresponds to the average of two platings per mouse.)

Effect of Virulence of the Strain on in Vivo Multiplication of Tubercle Bacilli Injected Intravenously into Mice.—

Mice were infected by the intravenous route with small doses ranging from 0.3×10^{-4} to 0.3×10^{-7} ml. of three strains of known degrees of virulence: MV, BCG-P, and BCG-T. Groups of three animals for each culture and for each infective dose were sacrificed 1, 2, 3, 5, and 7 weeks after infection, and the numbers of living bacilli present in the spleen and the lungs were determined by the usual techniques, with the exception that the oleic acid–albumin complex was replaced by a solution of plain albumin in the preparation of the solid medium for reasons given under Materials and Methods. The numbers of bacterial colonies recovered per individual organ are reported in Table VI.

With all infective doses of the three cultures, the numbers of living bacilli in the spleen increased up to a maximum and then decreased. For each individual culture the maximum was reached the earlier the higher the infective dose. For a given infective dose, the maximum was reached much sooner with the virulent culture MV than with the two BCG strains.

The numbers of bacilli in the lungs, at first much smaller than in the spleen, continued to increase throughout the period of observation, particularly in the case of the virulent strain. This was true even when the injected dose was so small (0.3×10^{-7} ml.) that it contained but a very few infective units.

It is important to note that however small the dose of BCG injected (0.3×10^{-6} ml.), there was some bacillary multiplication, particularly in the spleen. But in all cases it was slower and remained at a lower level than with the virulent culture, even when the size of the infective dose of BCG was 1,000 times greater than that of the virulent culture. Moreover, there were differences also between the two BCG cultures; it is of interest in particular that the strain (BCG-P) which reached the higher bacterial population level in the mouse was also the one which had been found in an earlier study to be capable of producing more severe lesions in the mouse lung and in the skin of guinea pigs (6).

It seems worth emphasizing again that the differences among the three strains tested held true over a very wide range of infective doses and were therefore an expression of some intrinsic properties of the cultures.

DISCUSSION

The pathogenicity of all the strains of tubercle bacilli used in the present study has been tested in the past by many different investigators under a variety of conditions. In agreement with general experience, it has been found in this laboratory that the strains H37Rv and Amerzanga (human type) and Ravenel and MV (bovine type) can induce in guinea pigs and mice a progressive and fatal disease. By contrast, the strains R1Rv (human) and BCG-P and BCG-T (bovine) produce in normal animals lesions which are self-limited

and regress naturally. These strains give rise to progressive disease only under very unusual conditions, for example in silicotic guinea pigs (2, 11). There are differences among these three strains of reduced virulence, and by comparing the duration and severity of the lesions which they produce it is possible to classify them in the following order of decreasing pathogenicity: R1Rv, BCG-P, and BCG-T. As to the strain H37Ra, it is not known to produce any significant lesions except when injected in large amounts (3–5, 7).

In the present study an attempt was made to measure the different levels of virulence by determining the numbers of bacilli which could be recovered from the various organs of mice following injection of known doses of the different strains. Subcutaneous and intraperitoneal injections of the bacilli proved unsuited for comparative tests, since the results were extremely irregular and because large doses were required to establish infection. By contrast, injection of even very small volumes of cultures by the intracerebral or intravenous routes was consistently effective, the results obtained by the intravenous route being the more reproducible from the quantitative point of view. It had already been reported by others that, following injection of large quantities of BCG vaccine into mice by the intraperitoneal route, only few of the bacilli reached the pulmonary parenchyma, far fewer than following intravenous injections (12).

The results presented in Table I show that the bacilli introduced by the intracerebral route immediately reached the blood stream and were thus distributed throughout the tissues. Then, they disappeared from the blood rapidly and permanently, probably through the agency of the clearing mechanism of the body. They could be recovered for long periods of time from all other organs which were tested (brain, spleen, lungs, and liver).

Only in the case of strain H37Ra was it impossible to find any evidence of multiplication *in vivo*. Whether injected intracerebrally or intravenously, the H37Ra bacilli decreased progressively in number in the spleen and the lungs. Injected intracerebrally, they did not multiply even in the brain. However, viable forms could be recovered from all these organs for many weeks—although in progressively decreasing numbers. It seems likely, therefore, that the bacilli were not being killed by humoral or cellular agencies, but died merely because the environment was not favorable for their reproduction.

With all the other strains, bacillary multiplication began in the spleen immediately after infection. But whatever the infective dose, the numbers of living bacilli that could be recovered from the spleen soon reached a maximum, and this was fairly characteristic for each strain. The maximum was higher and was reached sooner, the larger the dose injected and the more virulent the culture (virulence being defined here by the duration and extent of the lesions which the strain was capable of producing in guinea pigs). For

example, with the virulent culture MV, the maximum number of bacilli per spleen was reached within approximately 2 weeks after injection of 0.3×10^{-5} ml. of culture, and only after 5 weeks with 0.3×10^{-7} ml. inoculum. The maximum was lower and was reached more slowly with the two BCG strains than with the virulent strain MV even though the infective inoculum was larger in the case of the former strains. After the maximum was reached, the numbers of living bacilli in the spleen decreased slowly in the case of all strains, even when the infection eventually led to a fatal outcome. The numbers of bacilli in the lungs increased at first much more slowly than in the spleen. But with the virulent cultures in particular, they continued to increase in the lungs throughout the period of observation or until the death of the animal. These findings are consistent with those of earlier studies which have made clear that in mouse tuberculosis the lungs are much more affected than other organs—whether the extent of the disease be measured by pathological criteria or by the numbers of bacilli that can be seen in the organs on microscopic examination (4, 5, 13–16).

Considering the results as a whole, it appears that the strains used in the infection tests exhibited three contrasting types of behavior *in vivo*. Bacilli of the strain H37Ra survived for many weeks in the tissues of the mouse, but there was no evidence that their numbers increased at any time. They can therefore be considered as truly *avirulent*. Complete failure of multiplication has been observed also with another strain of human tubercle bacilli, namely, JH16Ra. It is of interest that these strains (and also R1Ra, a completely avirulent variant of R1Rv) fail to display in culture media the serpentine pattern of growth characteristic of the virulent forms, but grow instead in a helter-skelter, unoriented manner (5). It seems worth postulating that the cellular components or products which are responsible for the serpentine pattern of growth determine in some way the ability of the bacilli to multiply *in vivo*, but no conclusive experimental evidence in favor of this view has yet been presented.

Bacilli of strains R1Rv, BCG-P, and BCG-T underwent marked multiplication *in vivo* even when injected in very small amounts, but this multiplication came to a standstill after a period of 2 to 5 weeks. The numbers of living bacilli recovered from the tissues were to some extent a characteristic of the culture and the maximum was reached sooner, the larger the infective dose. Of particular interest is the fact that the two BCG substrains differed markedly in the extent of their multiplication *in vivo*. In fact, as will be shown in a later publication, the five BCG substrains studied in this laboratory have been found to differ in several morphological and biochemical characteristics, as well as in their behavior *in vivo*. All of them, like R1Rv, possess in common the ability to initiate an infection but this infection is self-limited. The multi-

plication of the bacilli is interrupted, probably by some cellular or humoral component, not necessarily of immunological character, brought into play by the tissue reactions which the bacilli evoke (17). Although BCG and similar strains are often referred to as "avirulent" because they never, or rarely, cause progressive disease and death, this appellation does not appear justified. Since they do multiply *in vivo*, they correspond in reality to what Pasteur designated as "attenuated" cultures, capable of producing an abortive infection.

The *virulent* strains differed from the attenuated in yielding more rapidly and with smaller infective doses, higher population levels in the various tissues. As in the case of the attenuated cultures, the numbers of living bacilli in the spleen increased only for 2 to 5 weeks, then decreased or remained stationary. But in the lungs, they continued to increase throughout the period of observation, soon reaching levels much higher than those found in the spleen.

The fact that bacilli of the attenuated strains R1Rv, BCG-P, and BCG-T undergo extensive multiplication in the mouse even though they cannot cause progressive disease is in keeping with observations made in rabbits infected with bovine and human virulent bacilli or with BCG (18). In the latter animal, only the bovine strains are fully virulent, yet inoculation with human bacilli or with BCG is followed by an initial phase during which these organisms multiply extensively in most organs. However, whereas multiplication of the bovine bacilli continues unchecked until death of the rabbit, the human bacilli soon stop multiplying, and BCG even sooner. Furthermore, bacillary multiplication of the human and the BCG strains in the rabbit is interrupted the sooner, the larger the dose of bacilli injected.

It is evident therefore that, like the virulent strains, the attenuated strains of tubercle bacilli are capable of initiating an infection in normal animals. Since failure of such strains to produce fatal tuberculosis is not due to the ability of the animal to prevent its initial multiplication *in vivo*, the control of the abortive disease must result from the mobilization of antimicrobial agencies of the tissues at the foci where infection has become established. It is probable that this form of resistance operates in part through the classical process of immunity—cellular or humoral. But it appears also from experiments now in progress in this laboratory that the inflammatory reaction creates in the diseased area a physicochemical environment which is inimical to the continued multiplication of the bacilli (17).

SUMMARY

Cultures of tubercle bacilli (typical bovine and human strains) known to differ in the severity of the lesions they induce in experimental animals, were injected in various doses into the cerebrum, peritoneal cavity, or blood stream of mice. Quantitative determinations of the numbers of living bacilli present

in the tissues at different intervals of time after infection led to the following classification of the cultures tested:—

(*a*) Certain well known variant forms of tubercle bacilli were found to be unable to multiply *in vivo*, although they could survive for many weeks in the tissues of mice. These organisms proved to be truly *avirulent*.

(*b*) Other variant forms underwent multiplication *in vivo*, even when extremely small infective doses were used, but could not give rise to progressive disease. It is proposed to designate these strains, which produce only abortive infections, as "*attenuated*." Different levels of attenuation could be detected. The maximum numbers of living bacilli that were recovered from the tissues corresponded directly to the severity and duration of the abortive lesions that could be produced by the strain in guinea pigs or in mice and were characteristic for each strain tested. The two BCG substrains tested were found to differ markedly in their level of attenuation.

(*c*) The cultures *virulent* for guinea pigs were also capable of establishing a progressive infection in mice even when small infective doses were used.

In the case of the attenuated and virulent strains, the population of living bacilli present in the lungs was at first much lower than that in the spleen, but it continued to increase in the former organs throughout the period of observation. This was notably true in the case of the virulent cultures. In contrast, the numbers of living bacilli in the spleen rapidly reached a maximum in the case of all cultures and then decreased progressively. For a given infective dose, and a given interval of time after inoculation, the maximum levels of living bacterial population attained in the spleen and in the lungs proved to be a direct expression of the virulence of the strain.

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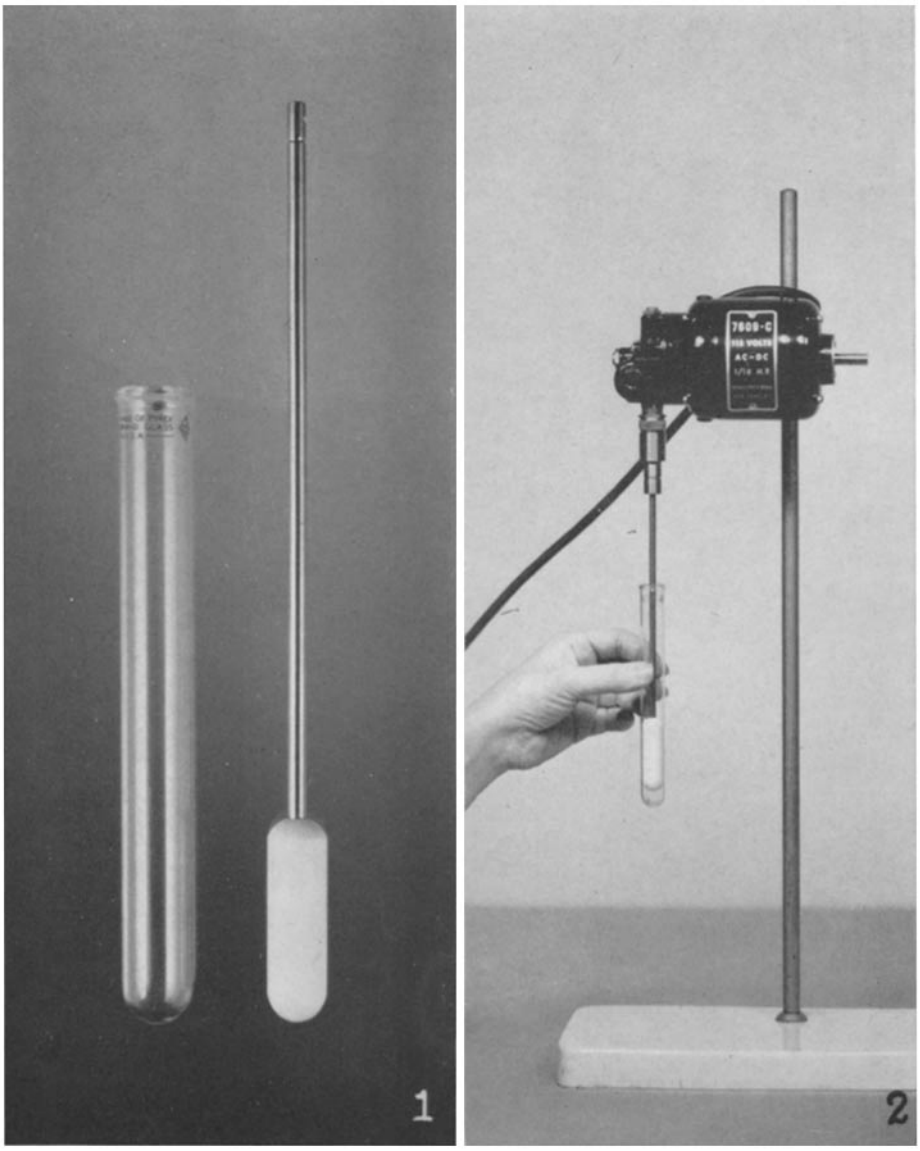
EXPLANATION OF PLATES

Photographs were made by Mr. J. A. Carlile.

PLATE 12

FIG. 1. Teflon homogenizer. × 0.5.

FIG. 2. Teflon homogenizer assembled for operation. × 0.2.

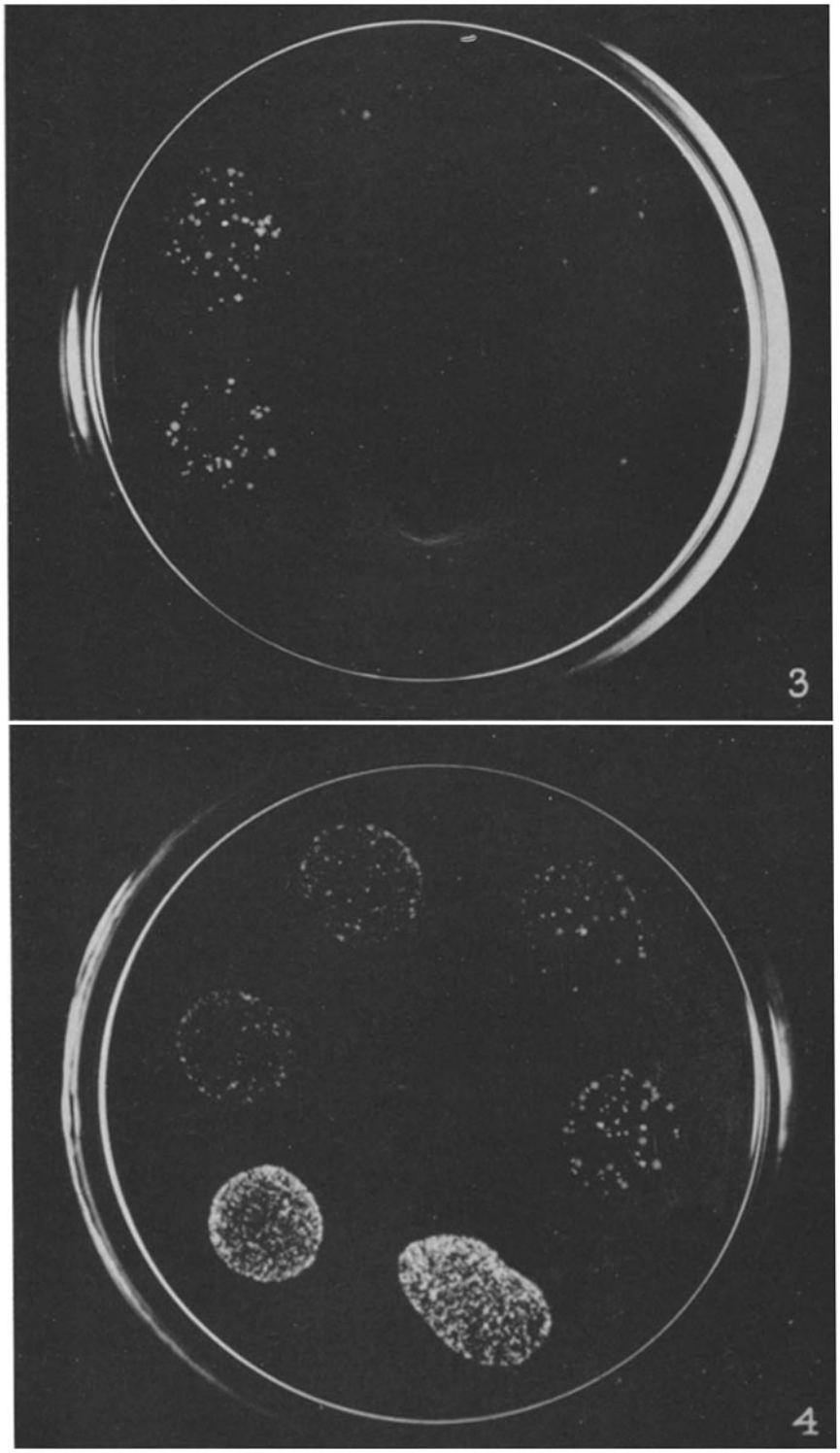


(Pierce *et al.*: Tubercle bacilli in mice)

PLATE 13

FIG. 3. Colonies of BCG-P on oleic acid–albumin agar inoculated 14 days previously with tenfold dilutions in duplicate of a suspension of infected mouse lung. See text. Actual size.

FIG. 4. Colonies of R1Rv on oleic acid–albumin agar inoculated 14 days previously with tenfold dilutions in duplicate of a suspension of infected mouse spleen. See text. Actual size.



(Pierce *et al.*: Tubercle bacilli in mice)