STUDIES ON TUBERCLE BACILLUS-MONOCYTE RELATIONSHIP*

IV. Effects of Passage in Normal and Immune Systems upon Virulent Bacilli

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There are various observations to indicate that under appropriate conditions of *in vitro* cultivation, attenuation of virulent tubercle bacilli may be achieved.

The BCG strain of tubercle bacillus, originally a virulent bacillus of the bovine type, provides a classical example of *in vitro* attenuation after prolonged cultivation on a glycerol-potato-bile medium (1). Similar reductions of bacillary virulence have also been accomplished by cultivation of virulent forms at low hydrogen ion concentrations (2, 3) and by growth beneath the surface of fluid media (4–6).

Although naturally occurring *in vivo* attenuation of virulent tubercle bacilli has been reported, particularly in association with lupus (7), it has been the general experience of investigators that experimental attenuation of virulent tubercle bacilli by *in vivo* methods was unsuccessful; even prolonged growth of mammalian tubercle bacilli in the bodies of cold-blooded animals has not resulted in any significant reduction of virulence (8).

As virulent tubercle bacilli which gain access into animal tissues are largely phagocytosed by mononuclear cells, it seemed a reasonable assumption that if destruction of bacilli or alteration of bacillary virulence were to occur, a propitious time for this would be during growth of bacilli in these cells. An earlier paper (9) has shown that passage of virulent tubercle bacilli in an immune system (monocytes of BCG-immunized rabbits cultivated in homologous immune serum medium) resulted in decreased capacity of bacilli to induce degeneration of normal monocytes.

The present paper reports the decreased capacity of tubercle bacilli to cause death of mice following passage of virulent bacilli in normal systems (normal monocytes maintained in normal serum medium) as well as in immune systems; it also describes certain other changes in the behavior of passaged bacilli.

Materials and Methods

Detailed descriptions of procedures and materials not previously used are presented below; those which were employed in earlier studies (9–11) are briefly described herein.

Monocytes.—Normal adult rabbits and rabbits injected intradermally with viable BCG

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were used as monocyte donors. Animals given BCG were not used as donors until 30 or more days after injection of bacilli at which time the animals were generally tuberculin-positive.

The monocytes were obtained by washing the peritoneal cavity of rabbits with chilled Tyrode's solution 5 days after intraperitoneal injection of 50 ml of klearol. The suspension of cells was centrifuged at 250 *g* for 3 to 4 minutes and the sedimented cells redispersed in 6 to 8 ml of freshly prepared 0.25 per cent trypsin in Tyrode's solution; the packed cells from the last washing were resuspended in a few milliliters of freshly collected normal or immune (BCG-immunized) rabbit serum, and the numbers of cells present were determined by counting in a hemocytometer. These cells were then used for preparation of passaged bacilli.

Nutrient Media for Cultivation of Monocytes.—The media used for cultivation of monocytes consisted of 50 per cent normal or immune rabbit serum in Tyrode's solution. Normal serum consisted of aged pooled sera from tuberculin-negative rabbits; immune serum was aged, pooled sera from tuberculin-positive rabbits which were immunized with BCG.

The pH of all media was adjusted to 7.4 with 5 per cent CO_2 in air before use.

Bacteria.—The bacteria used in these studies were the BCG, the H37Ra, and the H37Rv strains of tubercle bacillus.

The BCG strain of tubercle bacillus was cultivated on Calmette's potato medium. For use in immunization of animals which were employed as monocyte donors, the bacterial growth from a 2-week-old culture was ground with stcel balls, suspended in physiological saline, and diluted to contain 1.0 to 2.0 $\times$ 10^8 bacteria per ml. The amount of viable bacteria injected intradermally was 0.1 to 0.2 ml.

The H37Rv and H37Ra strains were grown in tween-albumin liquid medium for 6 to 7 days at 37°C. The cultures were washed several times in tween-albumin medium; after the last washing the sedimented bacteria were resuspended in a small volume of medium and centrifuged at 250 *g* for 3 minutes to remove larger aggregates. The supernatant fluids obtained in this manner were found to consist mainly of bacteria occurring singly; after determination of bacterial cell numbers in a Petroff-Hausser chamber under dark-ground illumination, the supernatant fluids were used for parasitization of monocytes or as a source of unpassaged bacilli.

Preparation of Passaged Bacteria.—Passaged bacilli consisted of 6-day-old cultures of the H37Rv strain which had been cultivated in (*a*) normal monocytes maintained in normal serum medium and (*b*) immune monocytes maintained in immune serum medium. Parasitization of these monocytes was accomplished by adding the H37Rv strain of tubercle bacillus to monocytes (approximately 10 bacilli per monocyte) in a screw-cap tube, centrifuging the mixture for 10 minutes at 850 *g*, and refrigerating for 1 hour at 4°C. After refrigeration the supernatant fluid was discarded and the sedimented cells washed with 5 ml of normal or immune serum medium; to remove the bulk of unphagocytized bacteria, the washed monocytes were sedimented and the supernatant fluid discarded. The washed cells were then resuspended in a small volume of the desired nutrient medium (normal or immune serum medium), counted in a hemocytometer, and diluted to yield 200,000 monocytes per ml; 5 to 6 ml of cell suspension were introduced into pyrex milk-dilution bottles and incubated for 6 days at 37°C. At the end of this period the infected monocytes were lysed by addition of saponin to a final concentration of 2 per cent. The chemical was allowed to act for 1 hour at room temperature and lysis was facilitated by repeated pipetting of the samples. The bacteria liberated from the infected monocytes were then concentrated by centrifugation, washed several times in tween-albumin medium, and finally resuspended in a small volume of the medium. When the bacterial suspension was found by microscopic examination to consist mostly of singly occurring bacilli, the numbers of bacilli were determined in a Petroff-Hausser chamber and viable counts were made at the same time.

Virulence Tests.—The Webster strain of Swiss albino mice (male, 4 to 6 weeks old) were inoculated intravenously with varying concentrations of passaged and unpassaged H37Rv prepared as outlined above. The animals were observed daily for deaths over a period of 6 to 8 weeks, and the infected tissues of dead animals were examined microscopically for presence of acid-fast rods.

Determination of Viable Bacilli.—The general procedure for determining the numbers of viable bacilli consisted of thorough dispersal of the starting material by repeated pipetting; microscopic examination of the material under dark-ground illumination was made and the samples were not used in determinations of viable bacilli until examination revealed primarily singly occurring bacilli. After thorough dispersal, dilutions of the sample were made in tween-albumin liquid medium and each dilution was added in triplicate to the surface of each of 4 glycerol-blood agar plates by the drop method (12). The plates were incubated for 12 to 14 days at 37°C and those plates which contained suitable numbers of colonies were counted and the average of all 4 plates calculated.

Neutral Red Test.—Passaged and unpassaged bacilli prepared in the manner described above were suspended in tween-albumin medium and sedimented by centrifugation at 2000 RPM for 30 minutes. The deposited bacteria were treated with 3 to 5 ml of 50 per cent methanol for 1 hour at 37°C. After this time, the bacilli were again centrifuged and the sediment extracted with a second portion of 50 per cent methanol. Following deposition of bacilli after the second exposure to methanol, they were resuspended in 3 to 5 ml of buffer solution (1 per cent sodium barbital and 5 per cent sodium chloride) containing neutral red; the test for ability of the bacilli to bind neutral red was read after 30 to 60 minutes at 37°C. (13).

Tests of Bacterial Susceptibility to Various Substances.—The general procedure for testing the susceptibility of passaged and unpassaged bacilli to various substances consisted of exposure of known numbers of viable bacilli to a given substance for varying periods of time and determination of the numbers of viable bacilli after exposure. The details for testing each substance were as follows:

Streptomycin.—Varying amounts of an aqueous solution of dihydrostreptomycin sulfate were added to tween-albumin media to yield final concentrations of 25, 50, and 100 μg/ml of medium. Known numbers of bacilli were added to these various media and the mixtures kept at 37°C.

Sodium Oleate.—Concentrations of sodium oleate varying by 10-fold steps were prepared by adding stock aqueous sodium oleate solution to tubes of Dubos media containing no albumin; the media were adjusted to pH 6.8–7.0 with sodium hydroxide solution before use. After addition of bacilli, the tubes were incubated at 37°C.

Monocyte Lysate.—Monocytes of normal rabbits were collected, washed, and counted in the usual manner; the cells were then lysed with distilled water (1.0×10^7 cells/ml) and the suspension lyophilized. When needed, the lyophilized material was reconstituted to its original volume with distilled water and passed through a glass filter. Varying amounts of filtrate were added to Dubos media containing no albumin. Bacilli were added to these media and incubated at 37°C.

EXPERIMENTAL RESULTS

Virulence of Passaged Bacilli for Mice.—The effect of passage in a normal or an immune system upon virulence of the H37Rv strain of tubercle bacillus is shown in the upper half of Table I. In comparing the virulence of passaged and unpassaged bacilli, it is apparent that bacilli grown for 6 days in normal or immune systems were of reduced virulence for mice. This was evidenced by the low mortality rates in animals given passaged bacilli; at the end of 6 weeks,

only 6 per cent of mice infected with bacilli from an immune system (monocytes of BCG-immunized rabbit cultivated in homologous immune serum) and only 22 per cent of mice infected with bacilli from a normal system (normal monocytes in normal serum) had succumbed; this is in sharp contrast to the 85 per cent mortality in mice receiving unpassaged bacilli (grown for a comparable period of time in tween-albumin medium).

The above differences in virulence between passaged and unpassaged bacilli did not result from injection of smaller numbers of passaged bacilli into animals

TABLE I

Virulence of Passaged and Unpassaged Bacilli for Mice

Type of bacilli inoculated*	Number viable bacilli inoculated ($\times 10^6$)	Total number mice inoculated	Per cent mortality (wks. after infection)			
			3	4	5	6
Unpassaged H37Rv	6.0	20	40	60	85	85
Normal-passaged H37Rv	13.0	18	6	6	17	22
Immune-passaged H37Rv	11.0	18	0	0	0	6
Unpassaged H37Rv (exposed to serum–cell lysate)	10.0	18	44	90	100	—
Unpassaged H37Rv	2.0	15	27	87	100	—
Intracellular H37Rv (from immune system)	1.5	14	0	0	0	21

* Unpassaged bacilli were grown in tween-albumin medium for 6 days. Unpassaged control consisted of unpassaged bacilli exposed to environment equivalent to that of bacilli passaged in immune systems; *i.e.*, in presence of serum medium, cell lysate, and saponin. Normal-passaged bacilli consisted of bacilli from normal rabbit monocytes cultivated in normal rabbit serum. Immune-passaged bacilli consisted of bacilli from monocytes of BCG-immunized rabbits cultivated in homologous immune serum. Intracellular bacilli were obtained from monocytes by thorough washing of infected monocytes and lysis of washed monocytes with saponin.

for, as shown in Table I, the numbers of viable passaged bacilli were approximately twice that of unpassaged bacilli.

The possibility that reduction of bacillary virulence may result from the manipulative procedures used in obtaining passaged bacilli (*e.g.*, adsorption by bacilli of inhibitory substances present in the environment) was tested by exposing unpassaged bacilli to conditions equivalent to those existing in cultures of infected immune monocytes (from BCG-immunized rabbits). The results of this test are shown in line 4 of Table I; it is evident that the constituents present in the environment (immune serum, immune cell lysate, saponin, salt solution) have no effect upon the virulence of tubercle bacilli, as evidenced by the 100 per cent mortality of mice inoculated with unpassaged bacilli which had been exposed to a serum medium–cell lysate mixture for 1 hour prior to their use in the virulence test.

The above findings may be interpreted in terms of modification of bacillary virulence during intracellular growth of bacilli. A direct test of this was made by thorough washing of infected monocytes (from an immune system) prior to lysis of cells by saponin; the intracellular bacilli released from these washed cells were then used in virulence tests. From an examination of the data in the lower part of Table I, it is quite clear that a population of bacilli of predominantly intracellular origin were of reduced virulence, for none of the infected animals died during the first 5 weeks and only 21 per cent succumbed at the end of 6 weeks. These results are in marked contrast to the 100 per cent mortality at 5 weeks of mice given an equivalent dose of unpassaged bacilli.

Repetition of these experiments (results not shown) have yielded data exhibiting the same general trend toward reduction of bacillary virulence following passage of bacilli in normal or immune systems. While the mortality rates varied somewhat from experiment to experiment, these rates in mice given passaged bacilli were consistently and markedly lower than those in mice infected with unpassaged bacilli. In one of these experiments where the observation period was extended to 8 weeks, it was found that the mortality rates in mice given passaged bacilli were at this time not significantly different from those observed at the end of 6 weeks.

Virulence of Passaged Bacilli After Growth on Laboratory Media.—To determine whether the state of reduced virulence of passaged bacilli was retained upon *in vitro* cultivation of passaged bacilli, two types of experiments were performed.

In the first type of experiment, normal-passaged bacilli or immune-passaged bacilli (passaged in normal or immune system) were introduced into tween-albumin liquid media (approximately 8.0×10^6 bacteria per ml of medium were added); these cultures were incubated at 37°C and when a fairly heavy growth was apparent (10 to 11 days), the bacteria were prepared in the manner described under Materials and Methods and used in virulence tests.

In the second type of experiment bacilli passaged in normal or in immune systems were injected intravenously into groups of 12 mice each. 4 weeks after infection surviving mice were sacrificed and their organs (liver, lung, spleen) pooled, ground, and used to prepare 20 per cent tissue suspensions; dilutions of the tissue suspensions were made and seeded on the surface of glycerol-blood agar plates; these were incubated at 37°C for 24 days and those plates showing good growth of isolated bacterial colonies were washed off with tween-albumin medium, thoroughly dispersed, and injected intravenously into mice. Unpassaged H37Rv was grown on the same medium for a comparable period of time and used in the virulence tests.

The results of *in vitro* cultivation of passaged bacilli in tween-albumin medium are shown in the upper half of Table II. It may be seen that when bacilli from normal or immune systems were subjected to growth in this medium, they

were no longer of reduced virulence; thus, the mortality rates in mice following intravenous injection of these bacilli were, at each of the recorded intervals of time, very similar to those observed for mice inoculated with unpassaged bacilli which had been grown in tween-albumin medium.

The results of virulence tests with passaged bacilli which were inoculated into mice and reisolated and grown on glycerol-blood agar plates are shown in the lower half of Table II. It is apparent that passaged bacilli treated in this

TABLE II

Virulence of Passaged Bacilli After Growth on Laboratory Media

Type of bacilli	Cultivation medium for bacilli	Number viable bacilli inoculated ($\times 10^6$)	Number mice inoculated	Per cent mortality (wks. after infection)			
				3	4	5	6
Unpassaged H37Rv	Tween-albumin*	4.3	12	17	33	58	75
Normal-passaged H37Rv	"	4.5	12	17	25	50	75
Immune-passaged H37Rv	"	9.0	12	17	33	67	83
Unpassaged H37Rv	Glycerol-blood agar‡	42.0	15	20	40	73	100
Normal-passaged H37Rv	Mouse glycerol-blood agar	85.0	15	20	60	80	100
Immune-passaged H37Rv	Mouse glycerol-blood agar	69.5	18	28	50	67	89

* The various types of bacilli were prepared in usual manner and then cultivated in tween-albumin medium for 10 to 11 days before use in virulence tests.

‡ Mice were inoculated intravenously with 2.0×10^7 normal-passaged bacilli and 1.4×10^7 immune-passaged bacilli; after 4 weeks, surviving mice were sacrificed and their organs (liver, spleen, lung) pooled, ground, and suspended in diluent to yield 20 per cent tissue suspension; dilutions of tissue suspensions were made and plated on glycerol-blood agar and incubated 24 days. The growth on plates containing abundant but isolated colonies was removed and placed in tween-albumin medium; after thorough dispersal of each bacterial suspension, virulence tests were made by intravenous injection of mice.

manner exhibited a high degree of lethality for mice. There were no significant differences in mortality rates caused by passaged and by unpassaged bacilli; for example, the mortality rates 5 weeks after infection were 67 and 80 per cent for immune-passaged and normal-passaged bacilli, respectively, and 73 per cent for unpassaged bacilli.

Effect of Passage upon Capacity to Bind Neutral Red.—Since the preceding results appeared to indicate that intracellular modification of bacillary virulence was not reflected in an inheritable change of the bacilli, the possibility that reduction of virulence occurred through modification of the bacterial cell surface was investigated. In view of the known difference between virulent H37Rv and avirulent H37Ra to bind neutral red, comparative studies of

passaged and unpassaged bacilli were made in terms of this capacity. As shown in Table III, passage of virulent H37Rv in normal or immune systems resulted in loss of ability to bind neutral red; passaged bacilli therefore resembled H37Ra and differed from virulent unpassaged H37Rv in this respect. It is also apparent from this Table that the loss of neutral red binding by passaged bacilli could not be duplicated *in vitro* by exposure of unpassaged bacilli to a serum medium–cell lysate environment, thus eliminating the possibility that loss of this capacity resulted from adsorption of environmental substances that caused interference with binding of this dye by virulent bacilli.

Effect of Sodium Oleate upon Passaged and Unpassaged Bacilli.—Alteration of the bacterial cell surface of passaged bacilli, as suggested by the loss of ability to bind neutral red, could conceivably be reflected in different be-

TABLE III

Neutral Red Binding by Passaged and Unpassaged Bacilli

Type of bacilli	Neutral red binding
Unpassaged H37Rv	Positive
Unpassaged H37Ra	Negative
Normal-passaged H37Rv	Negative
Immune-passaged H37Rv	Negative
Unpassaged H37Rv (exposed to serum-cell lysate)*	Positive

* Serum and monocytes of BCG-immunized rabbits were used.

haviors of these bacilli toward various inhibitory substances. This possibility was tested by studying the survival of passaged and unpassaged bacilli in the presence of a variety of deleterious agents. The results of these tests are reported in this and the following sections.

As shown in Table IV, exposure of either unpassaged H37Rv or H37Ra to a Dubos base medium containing sodium oleate (0.01 to 0.001 mg/ml) and tween but no albumin resulted in little or no loss of viable bacilli after 7 days at 37°C. Unpassaged H37Rv exposed to immune serum medium–cell lysate prior to use in the sodium oleate medium was likewise unaffected by the chemical. In contrast, bacilli from either a normal system or an immune system showed marked reduction in the numbers of viable bacilli after 7 days of exposure to sodium oleate. Thus, a concentration of 0.001 mg/ml of medium caused a drop of 1.1 log units of normal-passaged bacilli and a drop of 2.1 log units of immune-passaged bacilli. With 0.01 mg/ml of medium, there was a drop of greater than 4.1 log units of immune-passaged bacilli.

These results therefore indicated that unpassaged bacilli were relatively insensitive to the concentrations of sodium oleate used in these tests whereas

passaged bacilli, particularly those derived from an immune system, were highly susceptible to the action of this chemical.

A similar sensitivity of passaged bacilli to tween itself was demonstrated in some of our experiments (results not shown), but the results were less consistent in that the inhibitory effect of tween varied considerably with different batches of the chemical.

TABLE IV

Effect of Sodium Oleate upon Passaged and Unpassaged Bacilli

Type of bacilli	Concentration oleate	Log viable bacilli (days after incubation)*		Log decrease in viable bacilli at 7 days
		0	7	
	mg/ml			
Unpassaged H37Rv	0.01	6.7	7.2	0
	0.001	6.6	6.9	0
Unpassaged H37Ra	0.01	5.7	5.5	0.2
	0.001	5.7	5.8	0
Normal-passaged H37Rv	0.01	‡	‡	‡
	0.001	6.5	5.4	1.1
Immune-passaged H37Rv	0.01	6.4	<2.3	>4.1
	0.001	6.4	4.3	2.1
Unpassaged H37Rv (exposed to serum–cell lysate)§	0.01	6.8	7.0	0

* Incubation of bacilli was at 37°C in Dubos medium containing tween and sodium oleate, but no albumin.

‡ Sample not tested.

§ Serum and monocytes of BCG-immunized rabbits were used.

Effect of Monocyte Lysate upon Passaged and Unpassaged Bacilli.—It is evident in Table V that a lysate prepared from monocytes of normal rabbits (each milliliter of lysate contained the equivalent of 1×10^7 monocytes) had a bactericidal effect upon tubercle bacilli when bacilli were exposed to the lysate in a Dubos base medium containing tween but no albumin. Thus, in the presence of a 1:20 dilution of lysate, the numbers of viable unpassaged H37Rv were reduced by 2.5 log units. The inactivation of unpassaged H37Ra and passaged bacilli was even greater and amounted to 3.4 log units for normal-passaged bacilli, greater than 3.5 log units for unpassaged H37Ra, and more than 4.2 log units for immune-passaged bacilli. With a 1:80 dilution of lysate, the reduction in the numbers of viable unpassaged H37Rv was only 0.7 log

units and was in marked contrast to losses of 2.2 log units for normal-passaged bacilli and 3.2 to 4.2 log units for unpassaged H37Ra and immune-passaged H37Rv respectively.

These findings are thus suggestive of quantitative differences in the resistance of unpassaged and passaged H37Rv to the inactivating activity of normal monocyte lysate. In this respect, the marked sensitivity of passaged H37Rv to monocyte lysate resembled the behavior of unpassaged H37Ra.

Effect of Streptomycin upon Passaged and Unpassaged Bacilli.—The results

TABLE V

Effect of Monocyte Lysate upon Passaged and Unpassaged Bacilli

Type of bacilli	Dilution of monocyte lysate*	Log viable bacilli (days after incubation)‡		Log decrease in viable bacilli at 7 days
		0	7	
Unpassaged H37Rv	1:20	6.0	3.5	2.5
	1:80	5.9	5.2	0.7
Unpassaged H37Ra	1:20	5.8	<2.3	>3.5
	1:80	5.5	<2.3	>3.2
Normal-passaged H37Rv	1:20	6.6	3.2	3.4
	1:80	6.5	4.3	2.2
Immune-passaged H37Rv	1:20	6.5	<2.3	>4.2
	1:80	6.5	2.3	4.2

* Monocyte lysate was prepared to contain the equivalent of 1×10^7 monocytes/ml. Monocytes from normal rabbits were used.

‡ Bacilli were incubated at 37°C in a Dubos base medium containing tween and monocyte lysate but no albumin.

in Table VI show that when unpassaged H37Rv was kept for 12 hours at 37°C in tween-albumin medium containing streptomycin, considerable inactivation of bacilli occurred; the log decrease in viable bacilli ranged from 1.6 log units to more than 4.5 log units with concentrations of 25 to 100 μg streptomycin/ml of medium. When H37Rv was passaged in an immune system and tested against the same concentrations of streptomycin, the log decreases in viable count were fairly constant with all three concentrations of drug and amounted to 0.6 to 0.9 log units. This greater resistance of immune-passaged H37Rv to streptomycin did not result from adsorption of protective substances from the environment, for unpassaged H37Rv exposed to an immune serum–immune cell lysate system approximating that of passaged bacilli still proved as susceptible to the action of drug as untreated, unpassaged H37Rv. Un-

passaged H37Ra also proved highly susceptible to the action of streptomycin and its behavior seemed to resemble that of unpassaged H37Rv.

It would appear then that intracellular modification of virulent H37Rv did not necessarily result in increased susceptibility of bacilli to all inhibitory substances and that, in fact, it may lead to an increased resistance against certain agents such as streptomycin.

TABLE VI

Effect of Streptomycin upon Passaged and Unpassaged Bacilli

Type of bacilli	Concentration streptomycin	Log viable bacilli (hrs. after incubation)*		Log decrease in viable bacilli at 12 hrs.
		0	12	
	μg/ml			
Unpassaged H37Rv	100	6.8	<2.3	>4.5
	50	6.7	4.7	2.0
	25	6.7	5.1	1.6
Unpassaged H37Ra	100	6.3	<2.3	>4.5
	50	6.5	3.9	2.6
	25	‡	‡	‡
Immune-passaged H37Rv	100	5.8	4.9	0.9
	50	5.8	5.0	0.8
	25	5.7	5.1	0.6
Unpassaged H37Rv (exposed to serum–cell lysate)§	100	6.8	<2.3	>4.5

* Bacilli were incubated at 37°C in tween-albumin medium containing the designated amounts of streptomycin.

‡ Sample not tested.

§ Serum and monocytes of BCG-immunized rabbits were used.

DISCUSSION

The results of the present investigations have shown that growth of virulent tubercle bacilli in normal or immune systems (consisting of normal monocytes or monocytes of BCG-immunized rabbits and the corresponding serum) was attended by a marked reduction of bacillary virulence. The reduction of bacillary virulence, as evidence by decreased lethality for mice, was apparently the result of modification of bacilli in the intracellular environment, for a population of predominantly intracellular organisms obtained from thoroughly washed, infected immune monocytes proved highly attenuated for mice. It is conceivable, however, that lysis of infected monocytes by saponin with

subsequent release of bacilli into an environment containing all of the constituents of the tissue culture system may result in adsorption of inhibitory substances by bacilli with consequent decreased lethality for animals; this possibility was eliminated by the observation that exposure of unpassaged bacilli to the environmental constituents of the tissue culture system (immune serum, immune cell lysate, saponin, and salt solution) failed to effect a similar attenuation of the bacilli.

The studies detailed herein have shown that reduction of bacillary virulence was not the result of heritable alterations of the bacilli, since *in vitro* cultivation of passaged bacilli led to a reversion of virulence. On the basis of the loss of neutral red binding by passaged bacilli, it seems more likely that attenuation of virulent bacilli involved, at least in part, a non-transmissible modification of the bacterial cell surface.

The reduction in virulence and the loss of ability to bind neutral red were attended by changes in behavior of passaged bacilli toward inhibitory substances. Thus, passaged bacilli proved highly sensitive to inactivation by sodium oleate and by a lysate prepared from normal rabbit monocytes. In this respect, immune-passaged bacilli (*i.e.*, bacilli obtained from an immune system) seemed more sensitive than normal-passaged bacilli. Whether this was the result of a greater modification of bacilli by immune cells was not established in these investigations, but it is of interest to note than an earlier report (9) had demonstrated that immune-passaged bacilli, but not normal-passaged bacilli, failed to cause degeneration of normal rabbit monocytes; it is therefore a possibility that immune monocytes were more effective in modification of virulent bacilli and that the inability to demonstrate marked differences in mouse virulence between normal-passaged and immune-passaged bacilli in these studies may possibly be a reflection of the relative insensitivity of mice, as opposed to monocytes, to small differences in virulence.

The greater sensitivity of immune-passaged H37Rv to sodium oleate and to monocyte lysate was not observed with streptomycin, for immune-passaged bacilli proved more resistant than unpassaged H37Rv to the action of this drug. In this connection, it is of interest to note that certain isoniazid-resistant mutants isolated *in vitro* from laboratory strains of tubercle bacilli were also highly attenuated for guinea pigs (14).

The significance for the intact animal of the present findings of attenuation of virulent bacilli by intracellular passage in tissue cultures of monocytes remains to be determined. Segal and Block (15) have observed that bacteria grown *in vivo* in lung tissue proved more virulent for mice than those grown *in vitro*. The discrepancy between the present *in vitro* findings and the *in vivo* results of Segal and Bloch may possibly be due to the complexity of the *in vivo* environment. It is conceivable that if attenuation of virulent bacilli occurred during intracellular residence in the intact animal, its beneficial effects may not

always be apparent and, in fact, could be counterbalanced by various extracellular factors; thus, the possibility exists that in highly susceptible lung tissue, there may be conditions conducive to selection of more virulent bacilli. These are certainly problems that demand further analyses.

SUMMARY

Passage of the virulent H37Rv strain of tubercle bacillus in normal or immune systems (normal or immune monocytes suspended in the corresponding serum) resulted in decreased virulence of the bacilli; this was evidenced by the very low mortality rates in mice inoculated intravenously with passaged bacilli.

Passaged bacilli when cultivated directly in tween-albumin medium or when grown on glycerol-blood agar plates after recovery from infected mouse tissues proved as virulent as unpassaged bacilli.

The decreased virulence of passaged H37Rv was accompanied by loss of ability to bind neutral red.

Passaged H37Rv was more sensitive than unpassaged bacilli to inactivation by sodium oleate and by normal monocyte lysate; however, passaged H37Rv was more resistant than unpassaged bacilli to inhibition by streptomycin.

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