ANTITUBERCULOUS IMMUNITY IN MICE VACCINATED WITH KILLED TUBERCLE BACILLI

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It was shown in the preceding paper that antituberculous immunity could be detected in mice vaccinated with small amounts of living attenuated bacilli only after these bacilli had multiplied and reached a certain population level in the vaccinated animal (1). Two hypotheses can be invoked to account for this fact. It may be assumed on the one hand that tubercle bacilli produce *in vivo* certain substances which they do not produce under ordinary conditions of cultivation *in vitro*, and which are in some way essential for the development of resistance to infection. Anthrax is an example in point. Immunity to this disease in rabbits is the expression of an antibody against an "edema-producing factor" elaborated by the anthrax bacilli *in vivo*, but less readily *in vitro* (2, 3). Although it is well established that the metabolism and chemical structure of tubercle bacilli are profoundly affected by the environment in which they grow, there is as yet no experimental evidence suggesting that these organisms produce *in vivo* antigenic components different from those synthesized *in vitro*. This possibility, however, has not yet been eliminated.

A more simple hypothesis is that antituberculous immunity becomes established only after the amount of bacterial protoplasm present in the tissues is sufficient to cause a quantitatively adequate antigenic response; this situation is achieved when conditions permit extensive multiplication of the attenuated bacilli in the tissues of the vaccinated animal. If this second hypothesis were correct, it might be possible to immunize against tuberculosis by vaccinating with a sufficient amount of bacilli grown *in vitro* and killed by a technique designed to preserve the immunological activity of the protective antigen. Actually, many observers have shown that they could induce a certain level of antituberculous immunity in experimental animals by vaccinating them with bacilli killed by heat, formaldehyde, ultraviolet radiation, asphyxiation, etc. (4–14). Evidence of immunity has also been obtained when fractions separated from bacilli by chemical procedures have been used as vaccines (15, 16). At least three groups of investigators have reported some measure of success in the vaccination of human beings with killed bacilli (17–19). However, there have been also reports of failure too numerous to be listed here.

The discrepancy in findings and the conflicts of opinions among different groups of workers concerning the relative efficacy of living and killed vaccines make it necessary to reinvestigate the problem in order to determine whether differences in the conditions of experimentation are not at the root of the controversies. The present study was undertaken to define some of the factors which have to be controlled in order to increase the resistance of mice to tuberculous infection by vaccination with killed tubercle bacilli.

The cultures, media, and bacteriological techniques used in this study were the same as those described in a preceding paper (20).

Preparation of Suspensions of Bacilli Killed with 2 Per Cent Phenol.—Preliminary tests were carried out to compare the immunizing effectiveness of bacillary suspensions grown in different types of media and killed either by heat at different temperatures, or by treatment with formaldehyde or with phenol. These experiments will not be described here since they are to be extended in the future. Suffice it to say at this time that cultures of tubercle bacilli grown in tween-albumin medium and killed by the addition of phenol in a final concentration of 2 per cent, were found capable of eliciting in mice a limited but consistent immunity against virulent tuberculous infection subsequently induced. It was observed also that the vaccines appear to possess approximately the same immunizing activity irrespective of whether the bacilli used for their preparation had been cultivated in media containing glucose or glycerine as source of carbon. In most of the experiments to be described in this paper, the bacterial suspensions used as vaccines were prepared as follows:-

The various cultures were grown for 2 weeks in tween-albumin medium. Subcultures on a larger scale were then prepared by inoculating 10 ml. of these stock cultures into 100 ml. of the same medium, without albumin, but containing 0.5 per cent glycerine. After 2 weeks' incubation at 37.5°C. phenol was added to give a final concentration of 2 per cent. These "phenol-killed" cultures were kept at room temperature or at 37.5°C. for 48 hours, after which time the bacillary bodies were separated by centrifugation, washed twice in sterile distilled water containing 0.01 per cent tween 80, and resuspended in one-tenth the original volume of this same diluent. The bacillary suspensions thus prepared contained approximately 2.5 mg. dry weight bacilli per ml. and consisted of well formed acid-fast bacilli.

RESULTS

Comparative Immunizing Effectiveness of Killed Bacillary Suspensions Prepared from Strains of Various Degrees of Virulence.—It is shown in the preceding paper that the immunizing effectiveness of vaccines made up of living organisms is conditioned by the extent to which these organisms multiply *in vivo* (1). The following experiment was designed to test whether this characteristic of the strains was also reflected in the protective activity of the killed vaccines prepared from them.

Suspensions of phenol-killed bacilli were prepared from the six following cultures: H37Rv and MV (virulent); R1Rv, BCG-P and BCG-T (attenuated); H37Ra (avirulent). Single doses of 1.0, 0.2, and 0.04 mg. (dry weight) of these different vaccines resuspended in a volume of 0.2 ml. were injected intraperitoneally into 18 groups of ten mice each. Another group of ten unvaccinated control mice received 0.2 ml. of diluent. 2 weeks later all mice were challenged by the intravenous injection of 0.2 ml. of the virulent culture MV. A similar experiment was

carried out 1 year later, using only four cultures killed with phenol (MV, H37Rv, BCG-P, and H37Ra) and only one amount (0.5 mg.) of culture. As in the preceding case, the animals

TABLE I

Immunizing Effectiveness of Phenol-Killed Vaccines Prepared from Strains of Tubercle Bacilli of Various Degrees of Virulence

Strain from which vaccine was prepared	Dose of vaccine (dry weight)	Survival time of mice infected with 0.2 ml. virulent culture MV 2 wks. after vaccination										Average survival time
	mg.	*days*										*days*
MV (virulent)	1.0	10	14	17	17	17	23	23	24	24	30	21
" "	0.2	10	12	17	17	17	19	19	20	21	24	18
" "	0.04	10	11	12	15	15	15	17	17	17	17	14
H37Rv "	1.0	13	16	17	17	17	20	24	24	24	33	21
" "	0.2	12	13	13	17	17	17	17	21	24	25	18
" "	0.04	11	12	13	15	15	17	17	17	17	18	15
R1Rv (attenuated)	1.0	15	17	17	17	17	24	24	24	33	33	22
" "	0.2	12	13	13	15	17	17	18	19	24	24	17
" "	0.04	11	14	14	14	17	17	17	19	19	19	16
BCG-P "	1.0	16	16	17	17	17	24	24	34	34	34	23
" "	0.2	13	13	17	17	17	18	24	24	24	27	20
" "	0.04	10	13	14	14	17	17	17	17	19	19	16
BCG-T "	1.0	16	17	17	17	19	20	24	24	34	34	22
" "	0.2	12	14	17	17	17	20	22	24	24	26	19
" "	0.04	10	11	13	13	16	16	16	17	17	17	15
H37Ra (avirulent)	1.0	16	17	17	24	24	24	24	34	34	34	25
" "	0.2	13	15	16	17	17	18	19	24	24	24	19
" "	0.04	12	13	14	14	15	17	17	17	17	17	15
Controls (diluent)	0	11	12	12	13	13	15	15	17	17	17	14
MV (virulent)	0.5	12	12	21	21	22	27	27	27	27	27	25
			28	29	33	33						
H37Rv "	"	9	20	22	26	26	26	26	28	28	29	27
			29	29	33	33	36	36				
BCG-P (attenuated)	"	17	20	21	21	21	26	26	27	27	28	25
			28	29	30	33						
H37Ra (avirulent)	"	6	12	20	21	22	23	23	26	26	26	24
			29	29	30	30	33	35				
Controls (diluent)	0	6	6	6	12	12	15	15	16	16	17	14
			17	17	19	19	19	19				

The two experiments reported in Table I were carried out 1 year apart, with different batches of vaccines. This may account for the slight differences observed in immunizing capacity.

received the phenol-killed vaccine by the intraperitoneal route, and were challenged 2 weeks later by the intravenous injection of 0.2 ml. of virulent MV culture.

The results of these two experiments are presented in Table I.

The results presented in Table I show that mice which had been vaccinated with 1 mg. or 0.2 mg. of phenol-killed vaccine prepared from any of the six

cultures tested survived the challenge infection longer than did either the unvaccinated controls or the mice vaccinated with only 0.04 mg. of vaccine. There was no indication that the virulence of the culture was reflected in the immunizing efficacy of the phenol-killed vaccine prepared from it. It is of special interest that the vaccines prepared from strain H37Ra—which is unable to multiply *in vivo*—appeared as effective as those prepared from the virulent and attenuated strains.

As will be noted, there was much overlapping of survival time among the different groups, and for this reason, the results presented in Table I do not by themselves possess convincing value. They acquire significance only by virtue of the fact that a similar distribution of survival time between vaccinated and control groups was obtained in other experiments designed according to the same pattern. Attempts were made to develop an experimental procedure that would reveal more clear cut differences between the various groups of treated and untreated mice. As in the case of experiments dealing with the immunizing effectiveness of living vaccines (1), the method finally adopted consisted in infecting the test animals with a very small dose of virulent bacilli and determining at different intervals of time after infection the extent of bacillary multiplication in the spleen and lungs. The following experiments illustrate the effect of immunization with three different killed vaccines on the number of virulent bacilli found in the organs of mice 2 weeks after challenge infection.

Three groups of mice were vaccinated intraperitoneally by one single injection of 1.75 mg. of phenol-killed bacilli. The vaccines were prepared from bacilli of the strain MV (virulent) for one group, from BCG-P (attenuated) for the second, and from BCG-T (attenuated) for the third. A fourth group of mice served as control, the animals receiving the diluent instead of vaccine. 2 weeks later, ten mice of each of the four groups were challenged by the intravenous injection of 0.2 ml. of virulent living culture MV; the remaining four animals of each group received 0.2×10^{-5} ml. of the same culture. The survival time (in days) of the animals infected with the large dose of MV (0.2 ml.) is recorded in Table II. The mice infected with the smaller dose (0.2×10^{-5} ml.) were sacrificed 2 weeks after the challenge infection and dilutions of emulsions of their organs were plated on agar media; the numbers of bacterial colonies recovered from each organ are also recorded in Table II.

The results presented in Table II confirm the finding that mice vaccinated with phenol-killed bacilli (of either virulent or attenuated cultures) survived the large challenge infection longer than did the control mice. It is also evident that the state of immunity could be demonstrated by infecting the animals with a small dose of virulent bacilli. In this, and in most other similar experiments, the number of colonies recovered from the spleen of vaccinated mice was at least tenfold smaller than that recovered from control mice. Moreover, in confirmation of the results of the preceding experiment, all vaccines made up of phenol-killed tubercle bacilli were found to exhibit approximately the

same immunizing effectiveness irrespective of the virulence of the strain from which they had been prepared. For reasons of convenience, the vaccines used in all other experiments to be reported in the present paper were prepared from the avirulent strain H37Ra.

Effect of Dose of Vaccine on the Level of Protective Immunity.—Although vaccination increased the survival time of mice infected with large doses of virulent bacilli (Tables I and II), it did not prevent the lethal outcome of the infection. Similarly, vaccination decreased the multiplication of virulent bacilli used for the challenge infection in the organs of the immune mice, but it did not completely prevent their growth.

TABLE II

Evaluation by Two Different Techniques of the Antituberculous Immunity Induced in Mice by Intraperitoneal Injection of Phenol-Killed Vaccines

Strain from which vaccine was prepared	Dose	Survival time of mice infected intravenously with 0.2 ml. virulent MV 2 wks. after vaccination	Average survival time	No. ($\times 10^{-3}$) of colonies of tubercle bacilli recovered from mice infected intravenously with 0.2×10^{-5} ml. MV 2 wks. after vaccination							
				Spleen				Lungs			
	mg.	*days*	*days*								
MV	1.75	19 19 21 21 22 26 26	22	590*	28	24	19	61	17	3	0
BCG-P	"	13 19 19 19 19 20 20 23 23	21	910	700	650	127	600	94	49	15
BCG-T	"	19 19 19 19 19 19 19 21 21 23	20	73	47	—	—	130	6	—	—
Control saline	"	12 12 12 13 13 14 16 16 19 19	15	11,600	10,800	7,500	4,600	20,000	2,100	51	—

* 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.)

—The count was not available for technical reasons.

The following experiment was instituted to determine whether the level of immunity achieved was dependent upon the dose of vaccine used for immunization. The vaccine consisted of phenol-killed bacilli of the avirulent strain H37Ra. Its immunizing effectiveness was compared with that of living bacilli of the attenuated strain R1Rv.

Groups of mice were vaccinated by the intraperitoneal route with doses of phenol-killed bacilli of the H37Ra culture ranging from 0.075 to 1.5 mg., or with 0.2×10^{-2} ml. of the living R1Rv culture. Control mice received the diluent also intraperitoneally. 18 days later the animals were challenged intravenously by the injection of 0.2 ml. or 0.2×10^{-5} ml. of the virulent culture MV. The results of the experiment are presented in Table III.

It is apparent from the results presented in Table III that the phenol-killed vaccine prepared from the avirulent strain H37Ra prolonged the life of mice challenged with a large dose (0.2 ml.) of the virulent culture MV, and retarded somewhat the bacillary multiplication in the spleen of mice

infected with a small dose (0.2×10^{-5} ml.) of the same culture. But the level of immunity—as measured by either of the two techniques used—was essentially the same whatever the amount of vaccine injected. It was of the same order as that resulting from vaccination with living attenuated bacilli of the strain R1Rv.

It was not possible to test the immunizing effectiveness of doses of vaccine larger than those used in this experiment, because of the toxicity of the material for mice. The largest dose tested (2 mg.) was sufficient to prevent the animals

TABLE III

Effect of Dose of Phenol-Killed Vaccine on Protective Immunity

Vaccine	Dose intraperitoneal	Survival time of mice infected intravenously with 0.2 ml. MV 2½ wks. after vaccination	Average survival time	No. ($\times 10^{-3}$) of colonies recovered from spleens of mice injected intravenously with 0.2 $\times 10^{-5}$ ml. MV 2 ½ wks. after vaccination
	mg.	*days*	*days*	
H37Ra	1.5	10 21 21 21 21 21 22 24 28 28 28 30	22	16* 113 0 0
"	0.75	18 21 22 22 24 24 25 25 25 28 30	24	140 130 51 16
"	0.3	13 21 24 24 24 25 25 28 28 28 29	25	135 115 19 10
"	0.075	16 18 21 22 23 28 28 29	23	144 31 18 16
R1Rv living	0.2 ($\times 10^{-3}$ ml.)	13 13 16 21 21 21 21 22 23 24 24 28	21	147 47 19 6
Control	Saline	9 13 13 16 16 17 17 18 18 18 18 18 21	16	970 380 — —

—, the count was not available for technical reasons.

* 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.)

from gaining weight during the 2 weeks that followed vaccination; still larger doses (4 to 5 mg.) often caused death within a few days, a finding in agreement with that reported recently by Youmans and Youmans (21). The toxicity of the bacillary bodies was much the same, whether the culture was killed with heat or phenol. Experiments are in progress to test whether the vaccine can be freed of its toxic component without robbing it of its immunizing power.

Time of Appearance, and Duration, of the Immunity Induced by Phenol-Killed Bacilli.—Although no systematic study has yet been made of the variations with time of the immunity resulting from vaccination with killed bacilli, isolated observations indicate that the protective effect can persist for several weeks.

Mice were vaccinated by the intraperitoneal route with 0.75 mg. of phenol-killed bacilli of the H37Ra strain. An equal number of control animals received only the diluent. 16 days later, all animals were challenged intravenously by the injection of 0.2×10^{-5} ml. of the virulent culture MV. Groups of four mice vaccinated and unvaccinated were sacrificed 1, 8, 14, 32, and 56 days after the challenge infection (*i.e.* 17, 24, 30, 48, and 72 days after vaccination). The number of colonies of tubercle bacilli recovered from their spleens are reported in Table IV.

With the small infective inoculum used, bacilli could first be recovered consistently and in large numbers from the organs 14 days after the challenge infection. The numbers of bacilli in the spleen of the vaccinated animals remained smaller than in the controls throughout the whole period of observation but the difference was most striking at the 14 day period. These findings

TABLE IV

Duration of Immunity Induced by Intraperitoneal Vaccination with 0.75 Mg. Phenol-Killed Tubercle Bacilli (H37Ra)

Time after infection	No. ($\times 10^{-4}$) of colonies recovered from spleens of mice infected with 0.2×10^{-5} ml. MV							
	Normal mice				Vaccinated mice			
days								
1	0*	0	0	0	0	0	0	0
8	6	0	0	0	10	1	0	0
14	1,070	560	220	—	27	25	18	6
32	1,400	960	570	200	560	71	58	43
56	440	360	270	127	430	370	48	47

* 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.)

— The count was not available for technical reasons.

again emphasize the fact that, although the immune mechanism could retard bacillary multiplication, it was not capable of eradicating the bacilli from the infected organs.

In another experiment, mice were vaccinated with either 1.5 or 0.5 mg. of phenol-killed bacilli and were challenged 20 or 40 days later intravenously by the injection of 0.2×10^{-6} ml. of the virulent culture MV. They were sacrificed 3 weeks after infection. The numbers of colonies of tubercle bacilli recovered from their spleens and lungs are recorded in Table V.

The immunity against the very small infective dose used in this experiment appeared as effective 40 days as 20 days after vaccination. But it must be noted once more that the immune mechanism was unable to bring about the complete destruction of tubercle bacilli in the tissues since culture tests (not reported here) revealed that colonies could still be recovered from the spleen of mice sacrificed 5 weeks after the challenge infection.

Influence of Heat and of Certain Chemical Agents on the Immunizing Antigen.—A number of preliminary experiments have been carried out to determine the effect of various methods of treatment on the immunizing activity of bacillary suspensions, in the hope that the information thus gained would eventually help in developing better procedures for the preparation and purification of the effective antigen.

Bacilli of the H37Ra strain, killed with 2 per cent phenol, were resuspended in saline, or in 0.04 N HCl (final pH 2.0), or in 0.04 N NaOH (final pH 10.0). These bacillary suspensions were heated in a boiling water bath for 30 minutes, then brought back to neutral reaction before being used as vaccines.

In another experiment, approximately 100 mg. of desiccated cells of H37Ra was ground thoroughly in a mortar with 88 per cent phenol until the cells had lost their structure and

TABLE V

Duration of Immunity Induced by Intraperitoneal Vaccination with Phenol-Killed Avirulent Bacilli (H37Ra)

Vaccine	Time between vaccination and challenge infection	No. ($\times 10^{-4}$) of colonies recovered from mice sacrificed 20 days after challenge infection with 0.2×10^{-6} ml. MV					
		Spleen			Lungs		
mg.	*days*						
1.5	20	560*	280	220	17	0	0
0.5	20	800	117	85	0	0	0
1.5	40	280	79	0	210	220	0
0.5	40	530	220	190	1	1	0
0	Control	3,800	3,500	2,800	870	670	370

* 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.)

acid fastness. The thick suspension in phenol was centrifuged. The insoluble material was washed with acetone to remove the phenol, then desiccated, and finally resuspended in 0.01 per cent tween solution. The material soluble in 88 per cent phenol was precipitated by addition of 10 volumes of acetone, then desiccated, and finally redissolved in 0.01 per cent tween. These different materials were tested for immunizing activity in mice by intraperitoneal injection of amounts of each of the materials derived from 2.0, 0.8, 0.5, or 0.2 mg. of bacilli (dry weight). Mice vaccinated with the original suspension of phenol-killed bacilli, or with living BCG-T (0.2×10^{-4}) were included in the test.

Three or four mice of each group were infected intravenously with 0.2×10^{-5} ml. of culture MV and sacrificed 2 weeks later; the numbers of colonies of tubercle bacilli recovered from their spleens and lungs are reported in Table VI. In the first experiment some of the animals were challenged intravenously 2 weeks after vaccination by the injection of 0.2 ml. of virulent culture MV. The survival times are given in Table VI.

It appears from the results presented in Table VI that the immunizing activity of phenol-killed bacilli was greatly diminished by heating. In other

tests, not reported here, evidence of immunity was obtained by vaccinating with the culture heated at pH 10.0, but the results were erratic. Facts compatible with the view that the immunizing component of tubercle bacilli is more resistant at alkaline than at acid pH have been reported by others (7, 12).

TABLE VI

Influence of Various Treatments on Immunizing Efficacy of Phenol-Killed Avirulent Tubercle Bacilli (H37Ra)

Vaccine (intraperitoneal)	Mg.	No. ($\times 10^{-2}$) of colonies recovered from the spleens of mice sacrificed 2 wks. after intravenous infection with 0.2 $\times 10^{-6}$ ml. MV				Survival time of mice infected intravenously with 0.2 ml. MV 2 wks. after vaccination								Average survival time
						days								*days*
Phenol-killed (unheated)	0.5	420*	170	23		14	17	20	21	S‡	S	S	S	23
Heated in saline	"	2,220	1,140	65		13	14	15	16	20	24	27	27	19
" at pH 2.0	"	3,000	3,400	1,500		10	10	10	13	15	17	27	S	16
" " " 10.0, whole	"	4,500	1,300	740		11	14	14	15	15	16	20		15
" " " 10.0, soluble fraction from	"	4,000	3,000	1,890		6	7	8	8	9	10	10		8
Heated at pH 10.0, insoluble fraction from	"	2,400	1,100	680		10	14	15	15	15	16	17		15
Control (saline)	0	4,100	2,800	3,500		13	13	14	15	16	17	17	17	15
Phenol-killed (untreated)	0.8	780	470	160	80									
" " "	0.2	3,700	1,420	380	280									
Fraction soluble in 95 per cent phenol from	2.0	11,800	4,500	1,000	860									
Fraction soluble in 95 per cent phenol from	0.5	2,600	1,460	—	—									
Fraction insoluble in 95 per cent phenol from	0.8	430	260	250	160									
Fraction insoluble in 95 per cent phenol from	0.2	1,380	1,270	430										
BCG-T (living) 0.2 $\times 10^{-3}$ ml		270	112	22	20									
Control (saline)	0	7,300	4,100	3,600	960									

—, the count was not available for technical reasons.

* 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.)

‡ S indicates that animals were surviving when the experiment was terminated at the 28th day after the challenge infection.

The vaccine retained its protective activity after the bacterial cells had been disintegrated, and rendered non-acid-fast, by extraction with concentrated phenol. The active material was recovered in the fraction insoluble in 88 per cent phenol, whereas the cellular components extracted by these solvents were unable to induce in mice resistance to tuberculous infection.

DISCUSSION

Vaccination of mice with small amounts (1 mg. or less) of tubercle bacilli killed by exposure to 2 per cent phenol increased consistently their resistance

to experimental tuberculous infection. This increased resistance expressed itself in a somewhat prolonged survival of the animals following challenge infection with large doses of virulent culture, and more strikingly in the smaller numbers of bacilli that could be recovered from the spleens of mice infected with small doses of virulent culture. The protective effect persisted for several weeks (at least 7), but it was never sufficient to bring about the destruction of the infective bacilli. The degree of resistance afforded to the treated mice was of the same order as that induced by vaccination with living BCG or with other attenuated cultures.

Injection of phenol-killed tubercle bacilli into mice always caused marked splenomegaly, the spleen reaching a weight close to 0.5 gm. (instead of 0.1 gm. in the controls) and remaining enlarged for many weeks after vaccination. However, the increased resistance to infection did not seem to depend on this non-specific pathological effect. For, in many tests not reported in the present paper, a variety of other bacterial cultures, killed by heat or phenol, and used as vaccines in different amounts, failed to elicit in mice any degree of resistance to experimental tuberculous infection, although they caused much enlargement of the spleen; the phenol-killed tubercle bacilli heated at acid reaction also caused splenomegaly while proving totally unable to elicit protection. These facts, added to the persistence for many weeks of the protective effect induced by the active bacterial fraction, make it improbable that the increased resistance was the result of a non-specific stimulation of normal defense mechanisms. The conclusion appears justified at the present time that phenol-killed bacilli induced a state of increased resistance by virtue of their specific antigenic activity; but nothing is known of the nature of the immune mechanisms which were set in motion.

Needless to say, the observations described in the present paper do not prove that the kind of increased resistance induced by phenol-killed bacilli plays any part in acquired immunity to tuberculosis in man or even in animal species other than the mouse. There exist in the literature, however, a few indications which suggest that the findings described here are not unlike certain phenomena observed in other studies of antituberculous immunity. First should be mentioned the many reports that the resistance of guinea pigs, rabbits, and man to tuberculosis could be slightly increased by vaccination with *heat*-killed bacilli. Noteworthy also is the fact that partial immunity in guinea pigs could be induced by vaccination with living cells of the culture H37Ra (22–24). As this culture has lost the power to multiply *in vivo* and as the amount of living cells used by other workers for the vaccination of guinea pigs was per unit of body weight of the same order as the amount found effective for the vaccination of mice in the present experiments, it would appear that mice and guinea pigs respond in a similar manner to some antigenic component of avirulent tubercle bacilli.

It is true that in contradiction to these views, many investigators have found it impossible to induce any significant antituberculous immunity by using as antigens either killed bacilli or H37Ra. However, many of these negative results can be explained on the basis of the facts reported in this and the preceding paper. Vaccination with H37Ra differs in one fundamental character from vaccination with BCG. Since the BCG bacilli multiply extensively *in vivo*, minute amounts of vaccine are sufficient to cause the production in the tissues of amounts of antigenic material large enough to stimulate an adequate immune response. By contrast, vaccination with H37Ra can have a protective effect only if the amount of bacillary material injected—in the form of either living or dead bacilli—is sufficiently large to contain the necessary amount of effective antigen.

In many cases in which killed bacilli (of any strain) have been used for vaccinations in the past, it is probable that the immunizing antigen had been inadvertently inactivated during the preparation of the vaccine. As can be seen from the results presented in Table VI, the immunizing efficacy of the vaccine (in mice at least) is much decreased by heating, and indeed completely destroyed by boiling at acid pH. There are hints in earlier publications that bacilli killed with formaldehyde (14) or by heating at acid reaction (7) lose, in part, their ability to elicit immunity in guinea pigs or rabbits. In consequence, it is not unlikely that the failure of other workers to detect a protective effect following vaccination with killed bacilli was due in part to the techniques used for the preparation of the bacillary suspension.

As is well known, none of the techniques of antituberculosis vaccination available at the present time is sufficiently effective to completely protect experimental animals against an infection lethal for the controls. In general, vaccination achieves nothing more than prolonging the survival time following virulent infection. Such low levels of immunity can be made more clearly manifest by using a very small virulent infective inoculum and by following quantitatively the fate of the bacilli in the tissues of the control and vaccinated animals. It would seem that the bacteriological techniques used in the present paper have some merit in this respect. These techniques have revealed that in mice, the type and level of immunity resulting from vaccination with phenol-killed bacilli are of the same order as those obtainable by vaccination with living BCG or with other living attenuated cultures. Since immunity can be established by the injection of small amounts of dead bacillary substance, it follows that BCG vaccination may exert its protective effect, not through the agency of some mysterious forces set in motion by the living bacilli growing in the tissues of the vaccinated host, but merely by the production of an adequate amount of bacillary protoplasm during multiplication *in vivo*. Experiments are now in progress to determine the nature of the cellular component responsible for the protective effect of vaccination. Some of the findings reported here may serve as a guide for the preparation of an effective antigen.

In the present study three cultures of human origin (H37Rv, R1Rv and H37Ra) and three of bovine origin (MV, BCG-P, and BCG-T) were used for the preparation of phenol-killed bacilli. All yielded vaccines of approximately the same activity whatever their degree of virulence. In fact, the vaccine used in most of the experiments reported in this paper, was prepared from the strain H37Ra, which has lost the power of causing progressive disease, and even of multiplying *in vivo*. Clearly then, the antigenic factor responsible for the induction of the type of resistance under investigation is unrelated to the cellular component(s) responsible for the virulence of tubercle bacilli or for the peculiarity of virulent strains to display the serpentine pattern of growth (25). This protective factor must possess a fairly good antigenicity, for a single injection of a few tenths of a mg. of *whole* killed bacilli suffices to establish in mice a state of increased resistance which lasts for many weeks. It seems certain that only a small part of the material contained in the bacterial bodies is of significance in inducing the immune state since a fraction soluble in 88 per cent phenol could be removed from the vaccine without affecting its protective effectiveness. Although antigenicity persisted after the cells had been disintegrated and rendered non-acid-fast by grinding with 88 per cent phenol it was destroyed by heating, particularly at acid reaction. All these properties taken together make it likely that it will be eventually possible to separate the factor which elicits protective immunity from other components of the cell—certainly from those responsible for the serpentine pattern of growth of virulent cultures. It will be of particular interest to determine whether the killed bacillary bodies can be freed of their toxic constituents and of those which induce the state of tuberculin allergy, by techniques which preserve their protective effectiveness.

SUMMARY

The resistance of white mice to tuberculous infection could be increased by preliminary vaccination with small amounts of tubercle bacilli killed by contact with 2 per cent phenol. Vaccine prepared from a variant strain of human tubercle bacilli unable to multiply *in vivo* (H37Ra) proved as active as vaccines prepared from either virulent or attenuated strains.

The immunity induced by phenol-killed bacilli persisted for several weeks. Under the conditions of the experiments, however, it was never able to bring about the death of the virulent bacilli used for the challenge infection, even when the infective inoculum was very small. Its protective effect could be detected (*a*) by the increased survival time of mice infected with a very large dose of virulent bacilli, and particularly (*b*) by the lower numbers of bacilli present in the organs of mice sacrificed at various periods of time after injection of sublethal infective doses. Under the proper conditions of vaccination the immunity produced in mice by phenol-killed cells of avirulent bacilli was of the same order as that produced by BCG.

The protective antigen proved to be susceptible to heat, particularly at acid reactions. It retained its activity when the bacilli were disintegrated and rendered non-acid-fast by grinding with concentrated phenol. It remained in the insoluble cellular debris when the bacilli were extracted with 88 per cent phenol.

Reasons are presented to support the view that the antigenic components present in the tubercle bacilli (avirulent as well as virulent) killed with phenol play a significant part in several manifestations of increased resistance to tuberculosis.

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