ANTITUBERCULOUS IMMUNITY INDUCED IN MICE BY VACCINATION WITH KILLED TUBERCLE BACILLI OR WITH A SOLUBLE BACILLARY EXTRACT

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It has long been known that the resistance of experimental animals to infection with tubercle bacilli of the mammalian type can be slightly increased by vaccination with killed mycobacteria. Indeed, this method of immunization has been applied in man against tuberculosis (1–4) and in cattle against Johne's disease (5). The literature concerning observations made in experimental animals has been reviewed in a recent publication from this laboratory (6).

It was also shown in the same paper that a predictable degree of antituberculous immunity could be produced in albino mice with a vaccine consisting of mammalian mycobacteria killed by contact with 2 per cent phenol (6). The immunity was demonstrated by two independent techniques: (*a*) observation of survival time following virulent infection, and (*b*) determination of the number of living bacilli present in the organs at different periods of time after injection of a small infective dose. As far as could be judged, the protective activity of killed bacilli was independent of the virulence and colonial characteristics (serpentine or disoriented) of the strain from which they were obtained, and it did not decrease when the integrity of their cellular structure, and their ability to take the acid-fast stain, were lost as a result of mechanical disintegration or treatment with concentrated phenol. Although stimulation of the normal defense mechanisms of the host may have played some part in the increased resistance induced by vaccination with killed bacilli, there was evidence that specific immunological processes were also involved.

One of the difficulties encountered in the production of antituberculous immunity with whole killed bacilli arises from the toxicity of the vaccine, the injection of amounts only slightly larger than that required to elicit the immune response bringing about a rapid loss of weight. In mice, death may occur within a very few days after injection of 5 to 10 mg. by the intraperitoneal route. Evidence has been obtained recently that a large part of the primary toxicity—local and systemic—of the killed bacillary bodies resides in a fraction soluble in monochlorobenzene and insoluble in petrolic ether at low temperature. Information concerning the toxic properties of tubercle bacilli is reviewed in reference 7.

The results to be described in the present paper appear to support the view that the toxic properties of the bacilli and their ability to act as a protective antigen are independent characteristics. Extraction of the antigen in a soluble form has proven a difficult task. A number of experiments, not to be reported

here, have revealed that vaccination with cellular material soluble in aqueous solvents, acetone, or petrolic or diethyl ether, did not increase resistance to infection and indeed often increased susceptibility. Of the solvents tested, only methanol has been found to extract from the bacillary bodies a fraction apparently devoid of toxicity for mice, yet capable of eliciting in these animals a definite degree of antituberculous immunity. Although extraction with methanol constitutes an inefficient method of preparation of the protective antigen, the results obtained in mice appear worth reporting in view of the fact that they confirm and extend those obtained some 30 years ago in guinea pigs and rabbits by Nègre with a preparation that he called "antigène methylique" (8–10).

EXPERIMENTAL

Cultures.—The strains of tubercle bacilli used in this study have been described in earlier publications (11). For the preparation of the vaccines the cultures were grown for approximately 2 weeks in tween medium (12), containing 0.5 per cent glucose or glycerol, but no albumin. The medium was distributed in 50 ml. amounts in 250 ml. Erlenmeyer flasks and inoculated after sterilization with 0.5 to 1.0 ml. of actively growing cultures. The flasks were incubated at 37°C. on a rotary shaking machine which agitated the cultures continually at a speed of 80 R.P.M.

Cultures of the virulent strain (M.V.) to be used for the challenge infection tests were grown in the same medium for 5 to 7 days without agitation. Before use, all cultures were examined for purity by a modification of the Ziehl-Neelsen staining technique (13).

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 1 or 2 days after weaning, at approximately 4 weeks of age.

The diet of the animals before and during challenge consisted of pellets (Ralston Purina "fox chow") and tap water *ad lib.* Animals of only one sex were used in any one experiment, or, when this was not possible, equal numbers of males and females were distributed in each experimental group. The animals were housed in groups of 10 in metal cages or glass jars prior to infection, and in small cages accommodating one or two thereafter. They were vaccinated within 1 week after being received, always by the intraperitoneal route. They were observed daily after vaccination and weighed at 3 day intervals until the time of challenge infection.

The challenge bacilli were injected into the caudal vein in a final volume of 0.2 ml. Undiluted culture was injected in experiments designed to observe the effect of vaccination on survival time. 12 to 24 mice were employed in each of the groups for these survival experiments. A $1:10^5$ dilution of inoculum was used when it was desired to determine the rate of bacterial multiplication in the spleen. The enumeration of living tubercle bacilli in cultures and in the organs of infected mice was carried out by techniques reported in an earlier publication (11).

Preparation of Suspensions of Bacilli Killed with 2 Per Cent Phenol.—To cultures of tubercle bacilli grown for 14 to 17 days as described above, phenol (88 per cent) was added to a final concentration of 2 per cent. The phenolized cultures were held at room temperature from between 2 and 5 hours, with occasional manual shaking. The phenol was then removed by washing the cultures twice with distilled water and three times with acetone. The bacillary mass was transferred in acetone to crystallizing dishes, and this solvent was permitted to evaporate at room temperature. The bacillary mass was then ground finely with porcelain mortar and pestle, and stored over anhydrous calcium chloride or copper sulfate at 4°C.

Vaccine suspensions were made by mixing the desired amounts of bacilli with sterile distilled water, saline, or tween solution, and emulsifying the mixture with a teflon grinder similar to that used in the grinding of mouse tissues (11).

Adjuvant.—The adjuvant employed to enhance the efficacy of the vaccines was similar to that described by Freund (14). Two parts of an aqueous suspension of vaccine were dispersed in one part arlacel A (a mannide monooleate dispersing agent manufactured by the Atlas Co., Wilmington), and then mixed thoroughly by mortar and pestle with two parts of the mineral oil, bayol F. Arlacel and bayol were not sterilized; the mixture was prepared immediately before use.

This adjuvant was toxic for mice when injected intraperitoneally in quantities larger than 0.1 to 0.2 ml. It also enhanced the toxicity of tubercle bacilli to such an extent that amounts of killed bacillary bodies larger than 0.15 mg. could not be used safely with adjuvant. The toxicity expressed itself in weight loss, disturbances of the central nervous system, diarrhea, enlargement of the spleen, visceral adhesions, and occasionally death within 10 days following injection. It was noted that animals apparently recovering from the toxic effects of adjuvant, or of large quantities of bacillary bodies in saline, occasionally died shortly after challenge (within 3 days); these early deaths could not be ascribed to the tuberculous infection.

On the other hand, it was also observed that some of the animals which survived the toxic effect of large doses of vaccine or of adjuvant, not infrequently appeared to exhibit unusual resistance to virulent infection. In order to minimize the role of the non-specific protection which follows acute physiological disturbance in the evaluation of the protective effects of vaccines, no experiments are here reported in which the vaccination procedure caused severe toxic reactions.

Effect of Adjuvant on the Immunizing Effectiveness of Avirulent Tubercle Bacilli Killed with 2 Per Cent Phenol.—As shown in earlier reports from this laboratory (6, 11) the degree and duration of antituberculous immunity induced in mice by vaccination is conditioned by the amount of bacillary material acting as antigenic stimulus. In the case of living vaccines, the effective amount of antigen is determined by the extent of multiplication *in vivo* of the vaccinating agent; in the case of killed vaccines by the dose of bacillary material injected.

During the initial phase of the present study experiments were instituted to test the effect of adjuvant on the antigenicity of killed bacilli. To serve as a basis of comparison, the immunizing effectiveness of phenol-killed bacilli obtained from an avirulent human strain was first determined by injecting graded doses in suspension in an aqueous medium. A living vaccine made up of attenuated human bacilli (R1Rv) was also tested in the same experiment.

Single doses of 3.0, 1.0, 0.3, and 0.1 mg. (dry weight) of phenol-killed H37Ra were injected intraperitoneally into 4 groups of 18 mice each. The vaccine was suspended in 0.2 ml. of a 2 per cent tween 80 solution in saline. A fifth group of 18 mice received an intravenous injection of 0.2×10^{-2} ml. of a living, 6 day old culture of the attenuated human strain R1Rv in the same diluent. The control group of 17 unvaccinated animals received 0.2 ml. of the diluent intraperitoneally. 15 days after vaccination, all animals were challenged by the intravenous injection of 0.2 ml. of culture of the undiluted virulent bovine strain (M.V.). The results of this experiment are shown in Table I.

The results presented in Table I show that a protective effect against infection could be detected with 0.1 mg. of killed bacilli, the smallest amount used

in this experiment. Immunity was somewhat better with 0.3 mg. and best with 1 mg. of vaccine. No improvement of immunity was observed by increasing the dose to 3.0 mg. The protection bestowed by vaccination with the living, at-

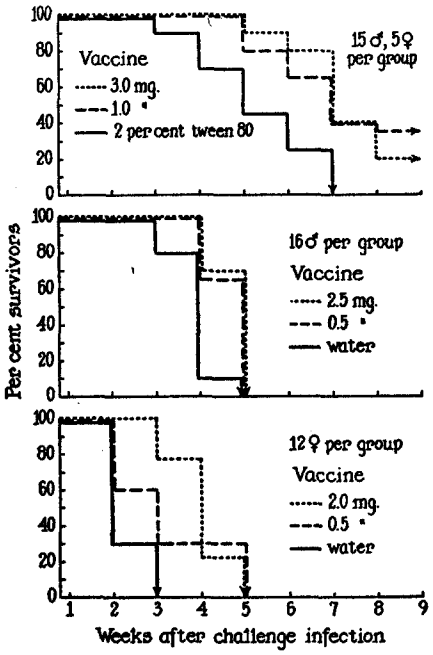

FIG. 1. Effect of vaccination with phenol-killed avirulent bacilli (H37Ra) suspended in aqueous diluents—(three experiments).

TABLE I

Effect of Vaccination with Graded Doses of Avirulent Bacilli (H37Ra) Killed with Phenol and Resuspended in an Aqueous Vehicle

Vaccine (intraperitoneal) in 0.2 ml. tween 80 (2 per cent)	Cumulative percentage of mice dead at following times after challenge infection				
	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.
H37Ra, killed 3.0 mg.	0	20	35	60	65
" " 1.0 "	0	10	20	60	80
" " 0.3 "	0	10	40	70	90
" " 0.1 "	15	35	65	80	100
R1Rv, living 0.2 × 10^{-2} ml.	6	30	50	60	100
2 per cent tween 80 (control)	30	55	75	100	

tenuated culture R1Rv was of the same order as that obtained with 1 mg. killed avirulent bacilli.

The results of three similar experiments in which groups of mice were vac-

cinated with different amounts of phenol-killed avirulent bacilli are illustrated in Fig. 1. It is seen that the degree of immunity was independent of the sex of the animals, the nature of the aqueous diluent in which the vaccine was administered (water, tween solution, or isotonic saline), and the severity of the challenge infection (as manifested by the difference in time of death of the unvaccinated controls).

Severe toxicity was consistently observed only when more than 2.5 mg. of killed H37Ra was injected. However, since mild toxic reactions were occasionally encountered with 1.0 to 2.0 mg., the optimum dose of vaccine was considered to be 0.5 to 1.0 mg. The following experiments show that addition of adjuvant to the vaccine permitted a 10- to 50-fold decrease in dose. This was demonstrated by determining the comparative ability of killed bacilli resuspended either in saline or in adjuvant to (*a*) prolong the survival time of mice following virulent infection and (*b*) retard the multiplication of virulent bacilli in the spleen of mice having received a small infective dose.

Single doses of 0.05 and 0.01 mg. (dry weight) of phenol-killed H37Ra were injected intraperitoneally in 0.1 ml. volume into four groups of 14 mice each. Two of the groups received the vaccine in 0.85 per cent saline; the other 2 were given the vaccine emulsified in bayol-arlacel adjuvant mixture. Two unvaccinated control groups of 15 mice each received 0.1 ml. of adjuvant or 0.1 ml. of saline respectively. 18 days later, all mice were challenged by the intravenous injection of 0.2 ml. of the undiluted virulent culture (M.V.).

In a parallel experiment, groups of 4 mice each were vaccinated intraperitoneally with 0.15 or 0.05 mg. of phenol-killed H37Ra in 0.2 ml. adjuvant mixture. Another group of 4 mice received an intravenous injection of 0.2×10^{-2} ml. of living BCG-T, and an unvaccinated control group of 4 animals was given an intraperitoneal injection of adjuvant alone. 18 days after vaccination, all animals were challenged by the intravenous injection of 0.2×10^{-5} ml. of the virulent culture. These animals were sacrificed 2 weeks after infection, and dilutions of emulsions of their spleens were plated on albumin agar medium. The results of these two experiments are presented in Table II (A and B).

The results presented in Table II A show that immunity could be elicited by very small quantities of killed bacilli when these were emulsified in adjuvant. In the survival experiment, significant protection was obtained with 10 μg. of phenol-killed H37Ra; smaller amounts have not been tested to date.

The consistency with which small amounts of tubercle bacilli elicit protection in mice when emulsified in adjuvant is shown in Fig. 2 which illustrates the results of four independent experiments.

The results of experiments designed to test the effect of vaccination on the multiplication of virulent bacilli in the spleen led to conclusions similar to those reached from the study of survival time. As shown in Table II B, the smallest quantity of vaccine (0.05 mg.) so far tested by the organ count technique decreased by 10-fold the number of virulent bacilli in the spleens of vaccinated mice in comparison with that found in control animals. Increasing the dose of vaccine to 0.15 mg. did not significantly better this degree of immunity, but caused toxic reactions.

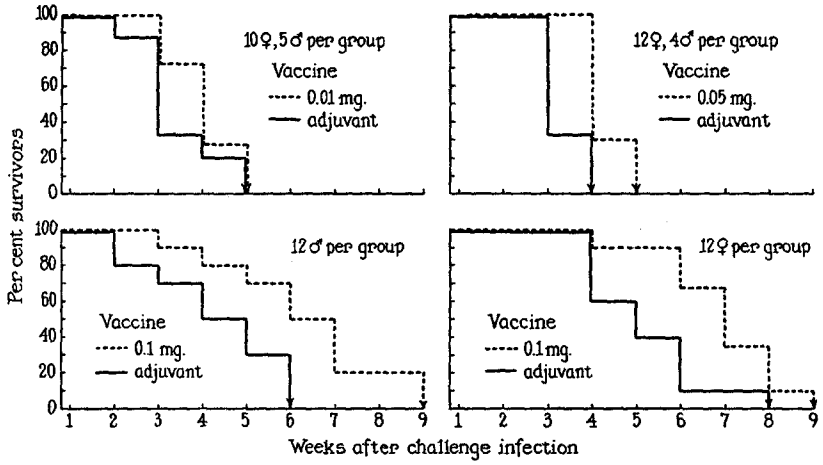

FIG. 2. Effect of vaccination with phenol-killed avirulent bacilli (H37Ra) emulsified in adjuvant—(four experiments).

TABLE II

Effect of Adjuvant on Immunizing Effectiveness of Avirulent Bacilli (H37Ra) Killed with Phenol

A

Vaccine (intraperitoneal)		Cumulative percentage of mice dead at following times after challenge infection			
Dose	Diluent (0.2 ml)	2 wks.	3 wks.	4 wks.	5 wks.
mg.					
0.05	Adjuvant	0	13	73	93
0.01	"	0	27	73	100
0 (control)	"	13	66	80	100
0.05	Saline	0	40	87	100
0.01	"	13	66	100	
0 (control)	"	13	66	93	100

B

Vaccine (intraperitoneal) in 0.2 ml. adjuvant		Nos.* of virulent bacilli recovered from the spleens of 4 mice 2 wks. after challenge with 0.2×10^{-6} ml. M.V.			
H37Ra, killed	0.15 mg.	66	54	23	22
" "	0.05 "	42	36	28	28
BCG-T, living	0.2×10^{-2} ml.	2.2	0.8	0.6	0.6
Adjuvant (control)	—	440	320	320	240

* Multiply by 10^3.

Since the adjuvant mixture is known to increase the persistence of certain antigens in animal tissues, an experiment was instituted to test its effect on the duration of the immune state elicited by killed bacilli.

Single doses of 0.1 mg. of phenol-killed H37Ra were injected intraperitoneally into two groups of 14 mice each. The vaccine given to one group was resuspended in 0.2 ml. distilled water, that for the other group in 0.2 ml. bayol-arlacel adjuvant. Non-vaccinated control groups of 12 animals each received 0.2 ml. of water or adjuvant respectively. 50 days later all animals were challenged with an intravenous injection of 0.2 ml. of the undiluted, virulent culture (M.V.). The results of this experiment are shown in Table III.

The results presented in Table III show that mice having received 0.1 mg. of phenol-killed H37Ra administered in adjuvant exhibited a high degree of

TABLE III

Effect of Adjuvant on the Duration of Immunity Induced by Vaccination with Avirulent Bacilli (H37Ra) Killed with Phenol*

Vaccine (intraperitoneal)		Cumulative percentage of mice dead at following times after challenge infection							
Dose	Diluent (0.2 ml.)	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.	9 wks.
mg.									
0.1	H_2O	10	10	20	50	80	100		
0	"	0	10	20	60	100			
0.1	Adjuvant	0	10	20	30	50	80	80	100
0	"	20	30	50	70	100			

* Challenge infection (0.2 ml. M.V.) given 50 days after vaccination.

immunity when challenged 50 days later, whereas mice receiving the same amount of vaccine in water died of the challenge infection almost as fast as the controls.[1] It has been shown in other experiments that the immunity elicited by vaccination with 1 mg. killed bacilli in saline begins to wane after 4 weeks.

It is thus apparent that the use of adjuvant not only increases the effectiveness of killed bacilli as protective antigen but also lengthens the duration of the immune state. Unfortunately, it is also true that the adjuvant increases the toxicity of the bacillary bodies to about the same extent that it increases their protective activity. This fact, which was to be anticipated from earlier findings (7), is further illustrated by the results of the following experiment.

Comparative Toxicity and Protective Effectiveness of Various Suspensions of

[1] The results also show a slight difference in the survival time of the unvaccinated controls receiving the two different diluents used to administer the vaccines. Consequently, the results in each vaccinated group should be compared with those in the control group which received the same diluent. (In this experiment, mice receiving only water survived somewhat longer than those given adjuvant. However, the opposite situation has been noted repeatedly in experiments in which challenge followed vaccination after the usual 2 week interval.)

Tubercle Bacilli.—Many attempts have been made in the course of this study to detoxify the bacterial suspensions while maintaining intact their protective activity—for example by treatment with formaldehyde. These efforts have been to no avail. They have revealed however that a number of chemical treatments which render the bacilli unable to elicit immune resistance in mice fail to decrease and indeed may even increase their toxicity. This is illustrated in the following experiment.

Tubercle bacilli of the H37Ra strain killed by exposure to 2 per cent phenol were subjected to the following treatments: One portion was heated to boiling for 15 minutes at pH 10. Another portion was similarly heated at pH 2.0. A third portion was suspended in 10 per cent formaldehyde and incubated overnight at 37°C., the pH being maintained at approximately 9.0. At the end of these treatments, the bacilli were washed in distilled water and

TABLE IV

Effect of Chemical Treatment on the Toxicity and Protective Activity of Avirulent Bacilli (H37Ra) Killed with Phenol

Vaccine (intraperitoneal) in 0.2 ml. Sesame Oil		Average weight change (gm. per mouse) at following times after vaccination			Cumulative percentage of mice dead at following times after challenge infection			
Treatment	Dose	3 days	6 days	17 days	2 wks.	3 wks.	4 wks.	5 wks.
	mg.							
Formaldehyde	2.0	−3.4	−1.6*	+1.4	0	20	90	100
100°C.-pH 10.0	"	−4.1	−2.1*	+1.1	0	30	90	100
100°C.-pH 2.0	"	−4.9	−3.5	‡	0	40	100	
Untreated	"	−3.6	−1.0	+1.3	0	20	70	100

* 1 dead.
‡ 3 dead.

dried. Single injections of 2.0 mg. (dry weight) of the bacillary material in 0.2 ml. sesame oil were given intraperitoneally to 3 groups of 12 mice each. A control group of 12 animals received an injection of 2.0 mg. of the untreated, phenol-killed bacilli. The animals were weighed immediately before vaccination, and again on the 3rd, 6th, and 17th day. 17 days after vaccination, all were challenged with 0.2 ml. of the undiluted virulent culture. The results of this experiment are shown in Table IV.

The results presented in Table IV show that the untreated preparation of killed bacilli was the most effective in eliciting protection and also the least toxic. Exposure of the bacillary mass to formaldehyde had little effect on either toxicity or immunity. Heating at pH 10 slightly decreased the immunizing capacity and increased the toxicity of the material. In confirmation of earlier findings (6), heating at pH 2.0 rendered the vaccine much more toxic, and less effective. Although further work with larger groups of animals will be necessary in order to define more accurately the effects of the manipulations on the vaccine, these results suffice to establish that the toxicity and protective activity of the bacilli are not similarly affected by chemical treatment.

Further evidence that the toxicity and the immunizing activity of the bacilli are the manifestations of different cellular constituents has come from a comparative study of two substrains of BCG (BCG-P and BCG-T). In two independent experiments, it has been found that, weight for weight, the killed cells of BCG-T are more toxic, and less effective as protective antigens, than those obtained from cultures of BCG-P grown for the same length of time under exactly the same conditions. This fact can be illustrated by the results of the following experiment.[2]

The strains BCG-P and BCG-T were grown under two different conditions: (*a*) as surface cultures in oleic acid–albumin liquid medium, or (*b*) as submerged cultures in liquid tween–albumin medium (both types of media are described in reference 12). After 4 weeks' incubation at 37°C., the bacterial cells were separated by filtration (surface cultures) or centrifugation (submerged cultures). They were killed by contact for 24 hours with 2 per cent phenol, then centrifuged, washed repeatedly with acetone-ether, and air-dried.

TABLE V A

Toxicity and Protective Activity of Cells of BCG-P and BCG-T Killed with Phenol

Strain	Vaccine growth conditions	LD_{50}	Average survival time following challenge infection*
		mg.	*days*
BCG-P	Surface	>20	21
" "	Submerged	>20	20
BCG-T	Surface	10	18
" "	Submerged	15	14
Control	—	—	12

* Mice vaccinated (intraperitoneally) with 2 mg. (dry weight) of bacillary suspension. Challenge infection (0.2 ml. M.V. intravenously) given 2 weeks after vaccination.

The acute toxicity was determined by injecting intraperitoneally 10, 15, or 20 mg. of bacillary material (resuspended in saline with a teflon grinder) into groups of 10 mice. The total number of deaths was recorded 7 days after injection (Table V A).

The immunizing effectiveness was tested by injecting intraperitoneally 2 mg. of the bacillary suspension (in saline) into groups of 16 mice each. The animals were challenged by intravenous injection of 0.2 ml. of virulent culture M.V. 2 weeks after vaccination. The average survival time of the vaccinated animals (in comparison with that of unvaccinated controls) is reported in Table V A.

In another experiment, the killed cells of the two substrains BCG-P and BCG-T were compared with regard to their toxicity and protective activity after the bacillary bodies had been extracted with monochlorobenzene. As previously shown (7), treatment with this solvent removes from the cells of BCG-P and other strains of tubercle bacilli a fraction possessing high toxicity for mice.

[2] This experiment was carried out in collaboration with Dr. Cynthia H. Pierce.

The cultures were grown for 4 weeks on the surface of liquid oleic acid–albumin medium (12). They were killed by exposure to 2 per cent phenol for 24 hours. The cells separated by filtration, washed with acetone, and air-dried were extracted four times with monochlorobenzene and four times with chloroform under the conditions described in reference 7. They were finally washed with acetone and air-dried.

For the toxicity determinations, single doses of 10.0 mg. (dry weight) of bacilli extracted with monochlorobenzene and chloroform, were resuspended in 0.2 ml. distilled water and injected intraperitoneally into 2 groups of 10 mice each.

For the determination of protective activity, 0.1 mg. (dry weight) amounts of extracted bacilli were emulsified in 0.1 ml. of the bayol-arlacel adjuvant mixture and injected intraperitoneally into 2 groups of 10 mice each. An unvaccinated control group of 10 mice received adjuvant alone. 14 days later, the animals were challenged by the intravenous injection of 0.2 ml. of the undiluted virulent culture M.V.

The results of this experiment are presented in Table V B.

TABLE V B

Toxicity and Protective Activity of Cells of BCG-P and BCG-T Killed with Phenol and Extracted with Chloroform and Monochlorbenzene

Vaccine (intraperitoneal)			Deaths following vaccination	Cumulative percentage of mice dead at following times after challenge infection					
Strain	Dose	Diluent (0.2 ml.)		4 wks.	5 wks.	6 wks.	7 wks.	8 wks.	9 wks.
	mg.								
BCG-P	10.0	H_2O	None						
"	0.1	Adjuvant	None	11	11	44	66	88	100
BCG-T	10.0	H_2O	7 of 10						
"	0.1	Adjuvant	None	10	60	100			
Control		"	None	40	60	90	90	100	

The results presented in Table V show that the phenol-killed cells of strain BCG-T were more toxic, weight for weight, than those of BCG-P. 10 mg. of the bacillary residue left after extraction of the cells of culture BCG-T with chloroform and monochlorobenzene killed 7 of 10 mice within 60 hours after injection, whereas no death occurred in animals which received the same amount of residue from the BCG-P strain. In contrast, both the whole and the extracted cells of the latter strain elicited a higher degree of immunity than did the vaccines prepared from BCG-T (Tables V A and V B).

Antituberculous Immunity Induced in Mice by a Methanol Extract of Phenol-Killed Avirulent Bacilli.—The results of the preceding experiment (Table V A and V B) reveal that BCG organisms extracted with monochlorobenzene and chloroform retain much if not all of their ability to elicit antituberculous immunity in mice. Many attempts have been made in this laboratory to extract in solution the bacillary component responsible for this activity. To this end, bacilli have been subjected to enzymatic digestion, extraction with various organic solvents, and other chemical procedures. Of the bacillary fractions thus obtained in solution only one so far has been found to possess some pro-

tective activity in mice. It has been obtained by exhaustive extraction of avirulent bacilli (H37Ra) with absolute methanol.

Preparation of Methanol-Soluble Extract.—Approximately 75 ml. of absolute methanol (reagent grade) was added to 1 gm. of phenol-killed bacilli. The mixture was placed in a round-bottom flask to which a water-cooled reflux condenser was attached. The apparatus was placed over a magnetic stirrer, in a water bath maintained at 55°C., with temperature variations not exceeding 1.5 degrees. A teflon-coated magnetic bar placed in the extraction flask with the mixture maintained the bacilli in suspension in the methanol with constant, but mild, agitation.

The bacilli were extracted for five periods of 6 hours each. At the end of each period, the methanol was decanted through two layers of filter paper and equal amounts of fresh solvent added to the suspension. The methanol extract was only slightly turbid while warm, but cloudiness increased rapidly upon cooling, and a white amorphous precipitate appeared after several hours at 4°C. This material usually redissolved completely upon heating the extract to 45°C. In order to avoid precipitation during filtration, the extract was filtered, while still warm, through paper maintained in a water-jacketed funnel heated to 65°C. Microscopic examination of the filtrate revealed no bacillary fragments.

Between periods of extraction, the bacillary mass was maintained statically in methanol at room temperature. Although the entire extraction took 72 to 96 hours, most of the soluble material was removed in the first two portions of solvent.

The five portions of methanol were pooled and evaporated to dryness over a 70°C. water bath. The residue was an amorphous, amber-colored, waxy material; it was immediately resuspended in warm (40°C.) distilled water or in 2 per cent tween solution. It was possible to prepare fairly homogenous suspensions containing 12 to 15 mg. of material per ml. by using material scraped from the walls of the evaporation vessel (usually a 600 ml. beaker) immediately upon complete evaporation of the methanol. If the extract was permitted to dry for periods longer than approximately 30 minutes, its color became brownish, and suspension in aqueous diluents could no longer be obtained. The extract was always used within 2 to 3 days after preparation, and usually within the first 24 hours.

It was not easy to prepare oil suspensions of the methanol extract. When used with oil-arlacel adjuvant, the material was first suspended in water and then mixed with the adjuvant.

Single doses of methanol extract (3.0 and 1.0 mg.) or of whole killed H37Ra (1.0 and 0.3 mg.) were injected intraperitoneally into four groups of 18 mice each. The vaccines were suspended in 0.2 ml. of 2 per cent solution of tween 80 in saline. A control group of 17 unvaccinated animals received 0.2 ml. of this diluent. 15 days after vaccination, all animals were challenged by the intravenous injection of 0.2 ml. of the undiluted virulent culture M.V.

In a similar experiment, single doses of methanol extract, (4.4 and 2.2 mg.) or of whole, killed H37Ra bacilli (2.5 and 0.5 mg.) were injected into four groups of 15 mice each, in suspension in distilled water. A control group of 15 animals received an equal volume of the diluent. The challenge infection took place 14 days later.

The results of these experiments are shown in Table VI.

The results presented in Table VI show that it is possible with the methanol-soluble extract to elicit a degree of protective immunity of the same order as that conferred by vaccination with whole killed bacilli. But this can be achieved only by administering amounts of the extract several times as large as those required of intact bacillary bodies.

The results of four independent experiments in which mice were vaccinated

with aqueous suspensions of the extract are illustrated in Fig. 3. As can be seen, immunity was obtained irrespective of age, sex, and severity of the challenge infection.

It is shown in the following experiment that vaccination with the methanol extract also proved able to retard the multiplication of virulent bacilli in the spleens of mice.

TABLE VI

Protective Activity of Avirulent Bacilli (H37Ra) Killed with Phenol and of Methanol-Soluble Fraction

A

Vaccine (intraperitoneal)			Cumulative percentage of mice dead at following times after challenge infection				
Preparation	Dose	Diluent (0.2 ml.)	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.
	mg.						
Methanol extract	3.0	2 per cent tween 80	0	15	45	60	90
" "	1.0	" " " " "	10	45	55	75	85
H37Ra, killed	1.0	" " " " "	0	10	20	60	80
" "	0.3	" " " " "	0	10	40	70	90
Control	0	" " " " "	30	55	75	100	

B

Vaccine (intraperitoneal)			Cumulative percentage of mice dead at following times after challenge infection			
Preparation	Dose	Diluent (0.2 ml.)	2 wks.	3 wks.	4 wks.	5 wks.
	mg.					
Methanol extract	4.4	H_2O	0	8	50	100
" "	2.2	"	0	0	30	100
H37Ra, killed	2.5	"	0	0	30	100
" "	0.5	"	0	0	35	100
Water (control)			10	20	90	100

Two groups of 4 mice each were vaccinated by the intraperitoneal injection of single doses of 1.0 mg. methanol extract or of 0.05 mg. whole, phenol-killed H37Ra bacilli. A third group of 4 animals received a mixture of 1.0 mg. extract and 0.05 mg. killed bacilli. All materials were injected in 0.2 ml. of the bayol-arlacel adjuvant; an unvaccinated control group received adjuvant alone. 18 days later, all animals were challenged by the intravenous injection of 0.2×10^{-5} ml. of the virulent culture. The animals were sacrificed 2 weeks after infection, and the number of living bacilli in their spleens were determined by quantitative bacteriological enumeration (11). The results of this experiment are shown in Table VII.

The results presented in Table VII show that vaccination with 1.0 mg. of methanol extract was as effective as vaccination with 0.05 mg. of whole bacilli

in retarding the multiplication of the virulent challenge organisms in the spleen. Other quantities have not yet been tested. The suppressive effect was enhanced when the extract was injected in admixture with whole bacilli.

The fact that large amounts of methanol extract were required to increase resistance against virulent infection indicates that the fraction extracted from

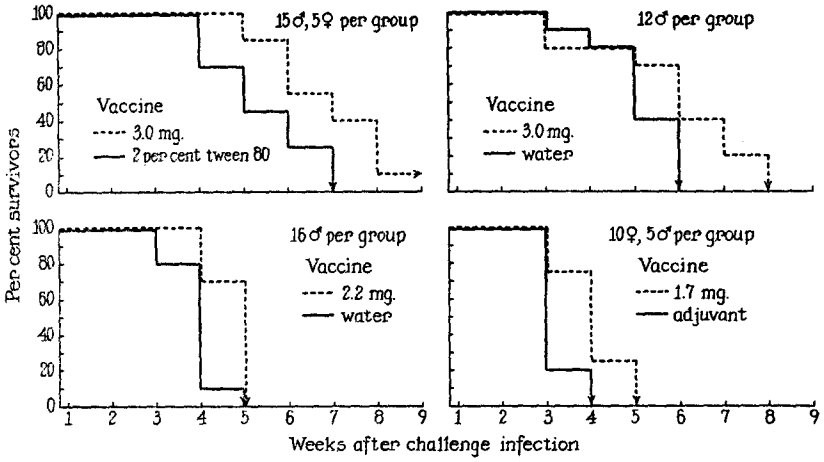

FIG. 3. Effect of vaccination with a methanol-soluble extract of phenol-killed avirulent bacilli (H37Ra)—(four experiments).

TABLE VII

Effect of Vaccination with Avirulent Bacilli (H37Ra) and Methanol Extract on the Multiplication of Virulent Bacilli in the Spleens of Mice

Vaccine (intraperitoneal) in 0.2 ml. adjuvant	No. of virulent bacilli* recovered from the spleens of 4 mice 2 wks. after challenge with 0.2 × 10^{-5} ml.			
Methanol extract 1.0 mg.	48	46	46	26
H37Ra, killed 0.05 mg.	42	36	28	28
Methanol extract 1.0 mg. + H37Ra, killed 0.05 mg.	9	8	6	6
Adjuvant (control)	440	320	320	240

* Multiply by 10^3.

the bacilli accounted for only a small part of their protective activity. Indeed, it is shown in the following experiment that the bacillary residue left after methanol extraction still retained much of the original activity.

Single doses of 2.0, 0.5, and 0.1 mg. of whole, phenol-killed H37Ra bacilli or of the cellular residue left after methanol extraction were injected intraperitoneally into 6 groups of 12 mice each. A control group received only the diluent (0.2 ml.). The animals were challenged by the intravenous injection of 0.2 ml. of virulent bacilli 17 days after vaccination. The results are presented in Table VIII.

The methanol extract did not cause any obvious toxic reactions in the amounts tested. The largest quantity injected—4.7 mg. intraperitoneally—was well tolerated by mice and did not even cause significant loss of weight. Autopsy of animals vaccinated with the extract revealed no, or only minimal,

TABLE VIII

Effect of Extraction with Methyl Alcohol on Protective Activity of Avirulent Bacilli (H37Ra) Killed with Phenol

Vaccine (intraperitoneal) in 0.2 ml. H_2O	Cumulative percentage of mice dead at following times after challenge infection				
	1 wk.	2 wks.	3 wks.	4 wks.	5 wks.
Whole killed H37Ra 2.0 mg.	0	0	22	88	100
" " " 0.5 "	0	40	70	70	100
" " " 0.1 "	0	50	100	—	—
Extraction residue 2.0 "	0	20	40	90	100
" " 0.5 "	0	50	100	—	—
" " 0.1 "	0	80	100	—	—
Water (control)	10	70	100	—	—

TABLE IX

Effect of Vaccination with Avirulent Bacilli (H37Ra) and Methanol Extract on the Weights of Mice

Vaccine (intraperitoneal) in 0.2 ml. sesame oil	Average change in weight per mouse relative to the pre-vaccination weight at following times after vaccination		
	4 days	7 days	13 days
	gm.	*gm.*	*gm.*
H37Ra, killed 3.0 mg.	−3.1*	−‡	−§
" " 1.0 "	−1.5	+0.5	+5.8
Methanol extract 3.0 "	−1.4	+1.2	+5.6
" " 1.0 "	−1.6	+2.0	+6.5
" " 0.3 "	−1.4	+1.0	+5.8
Sesame oil (control)	−1.7	0	+4.7

* 1 dead.
‡ 2 dead.
§ 3 dead.

visceral adhesions, whereas the intact killed bacilli caused extensive adhesions and spleen enlargement even when injected in small amount. Contrary to what happens with whole bacilli, emulsification of the extract with adjuvant did not appreciably increase its toxicity.

The effect on the weight of mice caused by injection of whole, killed bacilli or of methanol-soluble extract was tested in the following experiment.

Single intraperitoneal injections of whole, phenol-killed H37Ra (3.0 or 1.0 mg.) and of methanol extract (3.0, 1.0, or 0.3 mg.) were given to 5 groups of 13 mice each. The materials were injected in suspension in 0.2 ml. sesame oil. An unvaccinated control group of 13 mice received sesame oil alone. The animals were housed in metal cages and fed pellets and tap water *ad lib.* They were weighed at the same time of day (10 a.m.), on the 4th, 7th, and 13th day after vaccination. The weight changes relative to the weights of the animals taken immediately before injection are recorded in Table IX.

The results presented in Table IX show that intraperitoneal injection of 3.0 mg. phenol-killed H37Ra caused acute toxicity, 4 of the 13 animals dying within 13 days after infection. In contrast injection of 3.0 mg. of the methanol extract elicited no detectable toxic reactions.

DISCUSSION

It has not been judged necessary to report here the results of all the experiments carried out to test the protective effect exerted against mouse tuberculosis by vaccination with killed tubercle bacilli. Suffice it to state that in each of numerous tests carried out under many different conditions over the past 4 years, the average survival time of the vaccinated mice has been consistently and significantly longer than that of the control animals. The differences were never very large, and it is likely that all the vaccinated mice subsequently infected with massive doses of highly virulent cultures would have eventually died of tuberculous infection, had they not been sacrificed at an early date in order to terminate the experiments. The protective effect of vaccination could have been made to appear more impressive by using a less severe challenge infection, but in order to accelerate the progress of the study, it was deemed more practical to use an infective dose large enough to cause death of all controls within 10 to 30 days. It will be noted that, with this severe challenge infection, the immunity elicited by vaccination with living BCG was no more striking than that obtained with killed bacilli.

The toxicity of killed bacilli made it impossible to attempt enhancing the level of immunity by using a larger dose of vaccine. In fact, the range between minimum protective dose and maximum tolerated dose was not large. When the vaccine was administered by the intraperitoneal route in an aqueous vehicle, only little immunity could be detected with amounts smaller than 0.3 mg. of killed bacilli. Under these conditions, injections of more than 2 mg. caused the animals to lose weight and to appear ill; death frequently ensued when the dose was increased to 5 mg. Injection of the bacterial bodies in suspension in oil adjuvant decreased by a factor of 10 the minimum protective dose, but unfortunately increased toxicity in about the same proportion.

Yet there was evidence that the protective activity of the vaccine depended upon some factor other than the one responsible for toxicity. This appears from the comparison of the toxicity and protective activity of bacillary suspensions

prepared from various strains of mycobacteria. Thus, of two substrains of BCG handled under exactly the same conditions, one yielded a vaccine which was the more toxic, and the other a vaccine more effective as a protective agent. Other evidence supporting the dissociation between toxicity and protective activity is found in the fact—confirming observations reported earlier—that heating the bacilli at acid pH destroyed the latter property while increasing the former (6). Moreover, it has been found in many experiments to be reported elsewhere (15) that variation in the composition of the medium or in the conditions of growth (aeration, agitation, etc.) did alter independently the toxicity and protective activity of vaccines prepared from a given strain of tubercle bacilli.

The most convincing demonstration that protective activity and toxicity are two independent characteristics would be to achieve complete separation of the two properties by preparative procedures. As shown in another publication from this laboratory, one can separate from the bacillary bodies a highly toxic fraction soluble in monochlorobenzene, and insoluble in cold petroleum ether (7). In contrast, it has been found in the present study that extraction with methanol releases in a soluble form another bacillary fraction which has little if any toxicity, yet is capable of increasing the resistance of mice to tuberculous infection.

The existence in the bacillary bodies of a methanol-soluble substance capable of eliciting protective immunity was first pointed out over 30 years ago by Nègre (8–10). He resuspended living, virulent tubercle bacilli in methanol, 1 gm. of wet organisms per 100 ml. solvent, and incubated the mixture at 37°C. for 15 days; he termed the soluble fraction freed of the solvent, "antigène methylique." Injection of this material into guinea pigs or rabbits failed to render them allergic to tuberculin, yet was found to increase their resistance to infection with moderate doses of virulent tubercle bacilli.

The soluble fraction under consideration in the present study was prepared from a completely avirulent culture (H37Ra); it was released from dried, phenol-killed bacilli by thorough extraction with absolute methanol under continuous agitation for 28 to 30 hours at 55°C. The material thus obtained appeared to be of low toxicity for mice, the largest amount so far injected intraperitoneally—4.7 mg.—being without apparent effect on their general appearance, and depressing only slightly their weight curve. On the other hand, intraperitoneal injection of 1 mg. of this methanol extract increased appreciably the resistance of mice to infection. Indeed, the degree of immunity that it elicited was of the same order as that achieved by vaccination with living attenuated organisms or whole, killed bacilli. As the methanol-soluble fraction was free of bacillary fragments, its biological activity was due to some antigenic substance soluble in the methanol. It must be emphasized, however, that the method of extraction was either very inefficient, or destroyed much of the activity of the

protective antigen, for the extracted material accounted for only a small fraction of the total activity of the intact bacilli. Although five successive extractions with methanol released a total amount of soluble material corresponding to 10 to 15 per cent of the dry weight of the bacillary bodies, the bacterial residue still retained some 50 per cent of the initial protective activity after this treatment. Weight for weight, moreover, the extract was only about one-fifth as active as the whole bacilli.

In most experiments, the degree of antituberculous immunity elicited by vaccination was estimated by observing the survival time of vaccinated animals following injection of a very large challenge dose of virulent bacilli. This information was supplemented in a few tests by the quantitative determination of the number of bacilli present in the spleens of control and vaccinated animals 2 weeks after infection with a very small dose (0.2×10^{-5} ml.) of virulent culture. When evaluated by the spleen count technique, vaccination with large amounts of methanol extract yielded results of the same order as those obtained with killed bacilli; the number of virulent organisms recovered from the spleens of mice vaccinated with either of these two types of vaccine was some 10-fold smaller than that recovered from control animals.

As already pointed out, vaccination with living BCG did not increase the survival time of massively infected animals to a greater extent than did vaccination with killed bacilli or the methanol extract. There is no doubt however that BCG was superior to the killed vaccines in terms of duration of immunity and inhibition of the multiplication of virulent bacilli in the spleen of vaccinated animals. Many hypotheses can be formulated to account for these superiorities of BCG as an immunizing agent. It is possible for example that the living BCG organisms are distributed more effectively through the tissues of the vaccinated animal than are the killed vaccines, or that they produce a special protective substance while growing *in vivo*. On the other hand, it is very likely that some component of tubercle bacilli, while itself unable to elicit alone a protective antigenic reaction, can act as an adjuvant which enhances the effectiveness of a specific protective antigen. The latter hypothesis would provide an explanation for the fact that, weight for weight, the whole killed bacilli have greater protective activity than has the methanol extract separated from them. One might postulate that the methanol extract containing only the protective antigen(s) has little antigenic activity in the absence of the cellular constituents responsible for the well known adjuvant effect of tubercle bacilli. The toxicity of whole killed bacilli has so far made it difficult to put this hypothesis to an exhaustive experimental test. Progress will become more rapid if it proves possible to separate by chemical fractionation the various cellular components of tubercle bacilli and test them separately and in mixtures, both for toxicity and protective activity.

SUMMARY

It proved possible to increase the resistance of mice to tuberculous infection by vaccinating them with a suspension of avirulent tubercle bacilli killed by exposure to 2 per cent phenol. This increase in resistance was demonstrated by two different techniques: (*a*) observation of survival time of vaccinated animals following challenge infection with a large dose of virulent bacilli, and (*b*) determination of numbers of virulent bacilli in the spleens of animals 2 weeks after injection of a small infective dose.

The minimum protective dose of vaccine corresponded to approximately one-tenth the acutely toxic dose.

Addition of an adjuvant to the bacillary suspension markedly increased both the protective effectiveness of the vaccine and the duration of the immunity. It enhanced also the toxicity of the vaccine in approximately the same proportion. However, other lines of evidence suggested that toxicity and protective activity were independent one from the other and were the manifestations of different bacillary constituents.

Extraction with absolute methanol released from the bacillary bodies a crude soluble fraction possessing low, if any, toxicity, yet capable of eliciting in mice a state of increased resistance to virulent infection. The protective activity of this methanol-soluble fraction was low; it accounted for only a small part of the total protective activity of the original material.

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