ANTITUBERCULOUS IMMUNITY INDUCED BY METHANOL EXTRACTS OF TUBERCLE BACILLI—ITS ENHANCEMENT BY ADJUVANTS

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We have recently shown that the resistance of mice to experimental tuberculosis can be markedly increased by vaccinating the animals with material released from killed tubercle bacilli by extraction with methanol (15). This finding confirmed earlier reports of Nègre *et al.* concerning the protective activity in guinea pigs and rabbits of a fraction designated by them "antigène méthylique" (7–9). The present paper provides further illustration of the protective effect in mice of the methanol extract previously described. It also presents evidence that this protective effect can be enhanced and prolonged by the use of certain adjuvants.

EXPERIMENTAL

The bacteriological and chemical techniques used in the present study have been described elsewhere (15). The vaccination experiments were carried out with mice approximately 4 weeks of age which were fed pellets and water *ad lib.* throughout the period of experimentation. The results of infection tests are presented in the present paper in terms of cumulative percentage of deaths at given periods of time after infection. The size of the groups used for the calculation of these percentages ranged from 12 to 24 animals. Whenever both males and females were used, they were evenly distributed among the several groups of the particular experiment. There was no evidence of a difference in response between males and females to either the vaccination procedures or the challenge infection.

The methanol extract was prepared as described in the earlier part of this study (15). The tubercle bacilli were obtained by surface culture, killed with 2 per cent phenol, and washed with acetone. Two strains were used for the preparation of the extract: the avirulent strain H37Ra, and the attenuated strain BCG (substrain BCG-P). In general, methanol extraction was carried out at 55°C. as described; earlier in a few experiments, extraction took place at 27°C.

Comparative Immunizing Activity of Methanol Extract and of Whole Bacterial Bodies.—As shown in a preceding report, material capable of eliciting antituberculous immunity can be obtained by methanol extraction from the avirulent variant of human tubercle bacillus H37Ra; this variant grows in a completely non-oriented manner in artificial culture media (6, 13). It will be

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presently shown that a fraction possessing similar biological activity can be obtained also from a culture of BCG (BCG-P) exhibiting a serpentine pattern of growth (6, 11, 14).

The protective antigen soluble in methanol can be conveniently prepared from cultures of H37Ra which have been killed with 2 per cent phenol (15). A fraction corresponding to 10 to 14 per cent of the original cell material is thus released in a soluble form. It has been consistently observed, however,

TABLE I

Comparative Immunizing Effectiveness of Phenol-Killed Bacterial Bodies vs. Methanol Extract

Vaccine (i.p.) in 0.2 ml. distilled water		Cumulative percentage of mice dead at indicated times (days) after infection*					
Antigen‡		14	18	22	26	30	34
	mg.						
Bacterial bodies	7.5	33	33	55	77	88	88§
" "	3.0	0	0	18	45	45	54§
" "	1.0	0	0	24	56	64	64§
" "	0.5	0	24	32	64	80	80§
" "	0.1	0	40	48	72	88	88§
Methanol extract	10.0	8	16	48	64	72	72§
" "	4.0	0	0	32	48	72	72§
" "	1.3	8	8	56	64	72	72§
" "	0.7	0	32	56	88	88	88§
" "	0.13	8	32	88	88	100	
Control (water)		9	45	81	90	100	

* 0.25 ml. MV culture injected intravenously 19 days after vaccination.

‡ Both the bacterial bodies and the methanol extract were prepared from cultures of BCG-P.

§ Experiment discontinued 34 days after challenge infection.

that, weight for weight, the methanol extract of H37Ra possesses much less protective activity than the whole phenol-killed bacilli from which it is prepared. A similar situation has been found to obtain in the case of BCG. The comparative immunizing activity of the phenol-killed bacterial bodies and of the methanol extract prepared from this culture is brought out in the following experiment.

Eleven groups of mice (half male and half female) were vaccinated by the intraperitoneal route with graded amounts of phenol-killed cells of BCG-P (from 0.1 to 7.5 mg.) or of the methanol extract (0.13 to 10.0 mg.) prepared from another batch of the same culture. The vaccines were resuspended by grinding in saline and injected by the intraperitoneal route in 0.5 ml. volume. Control animals received saline alone. All animals were infected intravenously with 0.25 ml. of virulent culture MV, 19 days after vaccination (Table I).

The results presented in Table I show that the whole killed bacterial bodies were more effective than the methanol extract prepared from them in prolonging the life of mice infected with a large virulent dose. It took 4 mg. of extract to elicit a protection comparable to that obtained with 1 mg. of whole bacterial bodies.

In this experiment, the challenge dose was so large (0.25 ml. of virulent culture) that most of the unvaccinated animals died within 3 weeks after infection. It was therefore of interest to test the protective effect of vaccination in animals receiving a much smaller infective dose, and developing a disease with a more chronic course. In the following experiment a methanol

TABLE II

*Immunity Elicited by Killed Bacterial Bodies and Methanol Extract against Infection with Small Dose of Virulent Bacilli**

Antigen injected i.p. in 0.2 ml. distilled water		Cumulative percentage of mice dead at indicated times (months) after challenge infection*												
		1	2	3	4	5	6	7	8	9	10	11	12	13
	mg.													
Bacterial bodies‡	0.5	0	0	20	20	30	40	50	80	90	90	90	90	90§
" "	0.1	0	0	0	0	10	20	60	70	70	80	90	100	
Methanol extract‡	3.0	0	0	10	10	10	10	40	40	40	50	60	60	70§
" "	1.0	0	0	0	0	20	20	20	50	50	50	50	50	50§
Control (Water)		10	10	20	20	20	60	90	100					

* 0.0002 ml. of 10 day old MV culture in tween albumin medium injected intravenously.

‡ Both antigens were prepared from cultures of H37Ra killed with 2 per cent phenol and washed with acetone.

§ Experiment discontinued 14 months after challenge infection.

extract prepared from the avirulent H37Ra strain and the whole bacterial bodies of the same culture killed with phenol were used as vaccinating agents against an infection designed to cause a disease of several months duration.

Five groups of 20 mice each, all 3 week old males, were vaccinated by intraperitoneal injection of either one of the following antigens resuspended in 0.2 ml. water: 0.5 or 0.1 mg. of phenol-killed acetone dried, bacterial bodies of culture H37Ra; 3.0 or 1.0 mg. of methanol extract prepared from a similar batch of killed culture. The controls received water alone. All animals were challenged 15 days after vaccination by the intravenous injection of 0.0002 ml. of virulent culture MV (Table II).

As appears from the results presented in Table II, half the mice vaccinated with the methanol extract (either 1.0 or 3.0 mg.) were still living more than 1 year after infection when the experiment was discontinued, whereas most of the non-vaccinated animals had died within 6 months. With both amounts of extract, the protection against the small infective dose used in this test ap-

peared at least as good as that afforded by vaccination with the whole killed bacterial bodies.

Duration of Immunity Elicited by the Methanol Extract.—In the two experiments described so far, the challenge infection was administered a short time after vaccination (19 and 15 days respectively). The following experiment was instituted to determine whether the protective effect exerted by the methanol extract persisted beyond that short period of time. The duration of the immunizing effect was compared with that elicited by living BCG.

TABLE III

Comparative Immunizing Effectiveness of Living BCG and Methanol Extract

Vaccine injected i.p. in 0.2 ml distilled water	Cumulative percentage of mice dead at indicated times (days) after infection									
	18	22	26	30	34	38	42	46	50	54
	A. Challenge infection given 16 days after vaccination									
BCG-T, living* (i.v.)	0	0	16	16	40	48	72‡			
Methanol extract, 1.0 mg. (i.p.)	18	16	32	40	64	72	72‡			
Control (water) (i.p.)	16	48	72	72	80	88	88‡			
	B. Challenge infection given 62 days after vaccination									
BCG-T, living (i.v.)	8	8	16	16	16	64	80	80	80	80‡
Methanol extract, 1.0 mg. (i.p.)	0	8	16	16	16	16	32	40	48	88‡
Control (water) (i.p.)	0	8	16	32	40	56	72	80	80	88‡

* 0.002 ml. of 10 days old culture in tween-albumin medium injected intravenously.

‡ Experiments discontinued on 54th day after infection.

Two groups of 24 mice each (12 males and 12 females) were vaccinated, one by the intravenous injection of 0.002 ml. of a 10 day old living culture of BCG-T; the other by the intraperitoneal injection in 0.2 ml. of a suspension in water of 1.0 mg. of methanol extract (prepared from phenol-killed cells of BCG-P washed with acetone). A similar control group received 0.2 ml. of water alone intraperitoneally.

Twelve mice of each group were challenged intravenously with 0.2 ml. of virulent culture MV 16 days after vaccination. The other 12 mice of each group were similarly challenged 62 days after vaccination (Table III).

The results presented in Table III show that the protection elicited by the methanol extract was still evident when the animals were challenged 62 days after infection—at a time when the mice vaccinated with the living BCG had again become fully susceptible to tuberculous infection. It must be pointed out, however, that the BCG culture used for vaccination in this experiment (BCG-T) was one previously shown to disappear rapidly from the tissues of mice and therefore giving rise in these animals to an immunity of only short duration (1, 10).

Effect of Adjuvants on the Protective Activity of Whole Bacterial Bodies and of the Methanol Extract.—In all the experiments reported so far, the antigenic preparations under study were injected in suspension in water or saline. It has been shown elsewhere that the immunizing effect exerted by phenol-killed bacterial bodies can be markedly enhanced by injecting them in an oil emulsion (15). It will now be shown that the protective activity exerted by the methanol extract can also be somewhat enhanced by injecting it in association with certain adjuvants.

TABLE IV

Comparative Immunizing Effectiveness of Antigens Resuspended in Water or in Oil Adjuvant Mixture

Mixture injected i.p.		Cumulative percentage of mice dead at indicated times (days) after challenge infection*					
		14	18	22	26	30	34
	mg.						
Bacterial bodies‡	0.1 in water	0	40	48	72	88	88
" "	0.1 " adjuvant	0	8	24	48	56	80
Methanol extract	1.5 " water	8	8	56	64	72	72
" "	1.5 " adjuvant	0	9	27	36	45	54
Control (water)		10	45	80	90	100	
Control (adjuvant)		0	40	90	90	90	90

* 0.25 ml. of MV culture in tween medium, injected intravenously 19 days after vaccination.

‡ BCG cells killed with 2 per cent phenol.

Mice were vaccinated by the intraperitoneal route with either 0.1 mg. of phenol-killed acetone dried bacterial bodies of culture BCG-P washed with acetone, or with 1.5 mg. of methanol extract prepared from a similar killed culture. In the case of each antigen half the animals received the vaccine in suspension in 0.5 ml. of saline, and the other half in 0.2 ml. of an adjuvant, similar in composition to the Freund mixture (3). For this purpose two parts of arlacel A was emulsified with mortar and pestle, or by repeated mixing in a 5 ml. syringe, with one part of antigen suspension in water. This mixture was then emulsified with two parts of bayol F oil. Non-vaccinated control groups received either saline (0.5 ml.) or the adjuvant (0.2 ml.) alone. All animals were challenged by intravenous injection of 0.25 ml. of virulent MV culture given 19 days after vaccination (Table IV).

The results presented in Table IV make it clear that the protection elicited by either the bacterial suspension or the methanol extract was enhanced when the adjuvant mixture was used as vehicle instead of water. There was no indication that injection of the adjuvant alone had any protective effect.

Further evidence of the immunity-enhancing effect exerted by the adjuvant on small quantities of killed vaccine (0.1 mg. of whole bacterial cells) appears from a comparison of Tables II and V. These two tables present the results of experiments carried out simultaneously, with the same batches of antigens, the same challenge inoculum, and with mice of the same age (see description of experimental procedure given for Table II). The only difference was that in one case the antigens were administered in water (Table II), whereas the oil-arlacel adjuvant mixture was used as vehicle in the other case (Table V). It is apparent that the infected animals which had been vaccinated with the

TABLE V

Protective Effect of Antigens Emulsified in Oil Adjuvant against Infection with Small Doses of Virulent Bacilli

Vaccine injected i.p. in 0.2 ml. adjuvant		Cumulative percentage of mice dead at indicated times (mos.) after challenge infection*												
		1	2	3	4	5	6	7	8	8	10	11	12	13
	mg.													
Bacterial bodies‡	0.5	0	0	0	10	30	40	50	60	70	70	80	80	80§
" "	0.1	0	0	0	0	10	20	30	30	30	50	50	50	60§
Methanol extract	1.0	0	0	10	10	10	10	30	30	40	40	50	60	70§
Control (adjuvant)		0	0	0	10	30	60	90	90	100				

* 0.0002 ml. MV culture injected intravenously 21 days after infection.

‡ H37Ra cells killed with 2 per cent phenol.

§ Experiments discontinued 14 months after infection.

smaller amounts of bacterial bodies (0.1 mg.) survived somewhat longer in the series in which the antigen was injected in association with the oil adjuvant (Table V) than when water was used as vehicle (Table II).

It has been recently shown that the somatic polysaccharide of the typhoid bacillus is capable under the proper experimental conditions of enhancing the antigenicity of certain antigens, of certain proteins and of diphtheria toxin for example (4, 5). The following experiments provide suggestive evidence that this polysaccharide can also enhance the protective activity of the methanol antigen.

Four groups of female mice were injected intraperitoneally with the following materials in suspension in 0.2 ml. of water: (*a*) 1.1 mg. of methanol extract prepared from phenol-killed cells of BCG-P; (*b*) 30 μg. of a highly purified preparation of typhoid somatic polysaccharide generously supplied by Dr. M. Landy[1] of the Army Medical Service Graduate School, Washington, D. C.; (*c*) a mixture of 1.1 mg. of methanol extract and 30 μg. of ty-

[1] The authors wish to express their gratitude to Dr. M. Landy for calling their attention to the adjuvant effect of the purified somatic typhoid antigen and for supplying them with two samples of this valuable material prepared in his own laboratory.

phoid polysaccharide; (*d*) the controls received water alone. All animals were challenged by the intravenous injection of 0.2 ml. of a 6 days old culture of the virulent culture MV 41 days after vaccination (Table VI).

The results presented in Table VI show that the average survival time of mice vaccinated with a mixture of methanol extract and of typhoid somatic antigen was longer than that of any other group. In order to test the validity of this observation further experiments were instituted in which the typhoid somatic antigen was compared with other substances from the point of view of their ability to enhance the protective effect exerted by vaccination with the methanol extract. A sphingomyelin fraction from egg yolk and a bacterial

TABLE VI

Effect of Typhoid Polysaccharide on the Protective Effectiveness of Methanol Extract

Mixture injected i.p. in 0.2 ml distilled water		Cumulative percentage of mice dead at indicated times (days) after challenge infection*						
Methanol extract	Typhoid polysaccharide	20	24	28	32	36	40	44
mg.	*μg.*							
1.0	30	0	16	24	24	48	56	80‡
1.0	0	0	16	40	48	64	72	88‡
0	30	9	54	72	72	81	81	90‡
0	0	22	62	77	77	85	85	93‡

* 0.2 ml. MV culture injected intravenously 41 days after vaccination.

‡ Experiment discontinued 44 days after infection.

polysaccharide obtained from cultures of *Serratia marescens* were used in the following experiment.

Four groups of 24 mice each (12 male and 12 female) were vaccinated by the intraperitoneal route with 1.0 mg. of methanol extract prepared from phenol-killed cells of BCG-P culture. The mice of three of the groups also received respectively either 20.0 μg. of typhoid polysaccharide, or 40 μg. of pyromen (a polysaccharide preparation from *Serratia marcescens* distributed commercially by Baxter Laboratories, Morton Grove, Illinois) or 100 μg. of egg yolk sphingomyelin (obtained from the collection of the late Dr. P. A. Levene at The Rockefeller Institute). The fourth group received the methanol extract alone. In all cases, the materials were injected in suspensions in 0.2 ml. water; a fifth group of mice served as control and received water alone.

Half the animals of each group (6 males and 6 females) were challenged 16 days later by the intravenous injection of 0.2 ml. of virulent culture MV (Table VII A). The remaining 12 animals of each group were challenged with the same infective dose 62 days after vaccination (Table VII B).

The findings presented in part A of Table VII indicate that the typhoid somatic antigen enhanced the protective effect of the methanol extract against an infective dose administered 2 weeks after vaccination—thus confirming the

results of the preceding experiment. As shown in part B of the table, the adjuvant effect of the typhoid antigen manifested itself also in a prolongation of the immunity elicited by the methanol extract, marked protection being still evident when challenge infection took place 62 days after vaccination in mice having received both materials. Addition of pyromen to the methanol extract did not seem to affect the survival time of the vaccinated animals, whereas

TABLE VII

Comparative Effects of Pyromen, Typhoid Polysaccharide and Sphingomyelin on Protective Effectiveness of Methanol Extract

Mixture injected i.p. in 0.2 ml. distilled water			Cumulative percentage of mice dead at indicated times (days) after infection*									
Methanol extract	Vehicle		18	22	26	30	34	38	42	46	50	54
mg.		*µg.*	A. Challenge infection 16 days after vaccination									
1.0	Typhoid polysaccharide	20	0	0	24	32	40	64	88‡			
"	Pyromen	40	8	16	40	48	72	80	88‡			
"	Sphingomyelin	100	8	24	40	48	64	80	88‡			
"	Water		8	16	32	40	64	72	72‡			
0	Control (water)		16	48	72	72	80	88	88‡			
			B. Challenge infection 62 days after vaccination									
1.0	Typhoid polysaccharide	20	0	0	9	9	9	27	45	54	63	81§
"	Pyromen	40	0	0	0	20	30	40	70	80	80	100§
"	Sphingomyelin	100	0	0	0	8	8	8	32	32	40	64§
"	Water		0	8	8	24	40	64	64	72	96	
0	Control (water)		0	8	16	32	40	56	72	80	80	88§

* 0.2 ml. of MV culture in tween-albumin medium injected intravenously.

‡ Experiment discontinued 42 days after infection.

§ Experiment discontinued 54 days after infection.

mice having received sphingomyelin survived longer than those of any other group when challenged after 62 days. As the experiments with pyromen and sphingomyelin have not yet been repeated, the significance of the results obtained with these materials is questionable especially since no other concentrations have been tested. In contrast, the typhoid somatic antigen has been found to increase the resistance of mice vaccinated with methanol extract in three consecutive experiments. It seems worthwhile, therefore, to explore in greater detail and under a wider range of conditions the possible role of this substance as an adjuvant in experimental antituberculous immunization.

Comparative Toxicity of Methanol Extract and Whole Bacterial Bodies.—A

few remarks must be presented at this point concerning the toxicity of the preparations used for the vaccination of mice in the present study.

Examination of Tables I, II, and V makes clear that the level of protection afforded by vaccination with the whole killed bacterial bodies was very low when the amount of antigen injected exceeded a certain level. This fact has been traced to the toxicity of the bacterial suspension for normal mice as already brought out in earlier publications (2, 15). Similarly, there was some suggestion in the present study that vaccination with methanol extract was most effective when the amount of antigen injected (in water) did not exceed 3 gm.

TABLE VIII

Comparative Toxicity of Whole Killed Bacilli and Methanol Extract for Non-Infected Mice

Material injected i.p. in 0.5 ml. saline		Average change in weight per mouse at indicated times after injection	
		2 days	8 days
	mg.	*gm.*	*gm.*
Whole phenol-killed BCG	7.5	−2.0*	+1.0‡
" " " "	3.0	−1.6	+0.9§
" " " "	1.0	−1.0	+2.3
Methanol Extract	10.0	−0.8	+2.8
" "	4.0	+0.2	+2.8
" "	1.3	+0.3	+3.8
Control (Saline)		+0.6	+3.0

* 2 dead.
‡ 3 dead.
§ 1 dead.

A preliminary comparison of the toxicity of phenol-killed bacterial bodies (BCG-P) and of the methanol extract prepared from them, is presented in Table VIII. The results confirm that intraperitoneal injection of 1 mg. or more of phenol-killed acetone-dried cells of BCG-P (resuspended in saline) causes a rapid loss of weight in normal mice. Three animals out of six died within 8 days (two in 48 hours) after injection of 7.5 mg. of the material, and one with 3 mg. In contrast, even 10 mg. of the methanol extract prepared from the same suspension of killed BCG cells and resuspended in saline caused only very slight loss of weight in normal mice (Table VIII); this toxic effect was transient and none of the animals died. Similar toxicity experiments have been carried out with bacterial suspensions and methanol extract resuspended in various oil vehicles. In confirmation of earlier findings (15) it was found that the toxicity of the bacterial bodies under these conditions was some ten

times greater than when an aqueous vehicle was used for the injection. Oil also increased somewhat the toxicity of the methanol extract, but not to the same extent. It is clear, therefore, that on a weight basis the methanol extract is much less toxic than the total bacterial suspension, whether an aqueous or an oil vehicle is used for intraperitoneal injection. However, since the former material is also less effective as an immunizing agent, it has not seemed advisable to pursue further the comparison of the immunogenicity—toxicity ratios of the two antigens until the fraction of the methanol extract responsible for protective activity has been better defined.

DISCUSSION

The resistance of mice to experimental tuberculous infection can be consistently increased by vaccination with the methanol-soluble fraction extracted from killed tubercle bacilli. This fact has been established in more than twenty consecutive experiments carried out over the past 3 years under many different conditions in this laboratory. In all these experiments, the methanol extract has been injected intraperitoneally and the challenge infective dose introduced by the intravenous route. Active material has been obtained by extracting either surface growths of tubercle bacilli washed with water, but otherwise untreated, or cells previously killed with 2 per cent phenol and then washed with acetone. In the experiments reported in this and the preceding paper, the methanol extract was prepared from the cells of a BCG culture as well as from the avirulent strain H37Ra by extraction at 55°C. It will be recalled, on the other hand, that Nègre and Boquet had obtained their "antigène méthylique" by extracting the cells of virulent human tubercle bacilli for 2 weeks at 37°C (7–9). Moreover, these authors carried out their protection experiments in guinea pigs and rabbits. It is clear therefore that a fraction soluble in methanol and capable of increasing the resistance of laboratory animals to experimental tuberculosis can be separated from many strains of tubercle bacilli under a very wide range of conditions.

The results presented in this and the preceding paper (15), as well as more recent findings to be published later, indicate that the level of immunity that can be elicited in mice by vaccination with the methanol extract is of the same order as that resulting from vaccination with living BCG or with whole phenol-killed bacterial cells. It must be emphasized, however, that on a weight basis the protective activity of the methanol-soluble fraction is extremely low. In our experiments, treatment with methanol released in solution 10 to 14 per cent of the weight of the phenol-killed acetone-washed bacterial cells; yet it took 2 to 4 mg. of the methanol extract to elicit the degree of immunity that resulted from vaccination with 0.5 to 1.0 mg. of bacterial bodies.

The low protective activity of the extract may result of course from the inadequacy of methanol as a solvent of the active material. In fact, recent experiments have revealed that it takes many repeated extractions with

methanol to exhaust the bacterial cells of their protective ability. It is possible also that much of the protective material is inactivated in the course of extraction. Further light will certainly be thrown on these problems by the use of organic solvents other than methanol. The thought comes to mind on the other hand that part of the difference in activity between the total cells and the methanol-soluble fraction might be due to the adjuvant effect exerted by the cellular constituents of tubercle bacilli. No direct evidence supporting this hypothesis has yet been obtained, but it has been found that the protective activity of the methanol extract can be enhanced and prolonged by injecting this material in admixture with certain substances known to act as adjuvants in other immunological systems. Thus the minimum protective dose of methanol extract can be reduced several fold by using oil-arlacel mixture instead of water as a vehicle for intraperitoneal injection of the extract. In three consecutive experiments, an adjuvant effect could also be obtained by adding to the methanol extract small amounts (20 or 30 μg.) of a highly purified preparation of the somatic antigen of typhoid bacilli. Although experiments designed to compare the efficacy of various adjuvant mixtures are still in a preliminary state, they leave no doubt that it is possible by the use of these substances to extend over several months the protective effect achieved by vaccination of mice with the methanol extract of tubercle bacilli.

As shown elsewhere (15) and in the present paper, it is possible to enhance also the protective effect of whole phenol-killed bacilli by injecting them in association with oil-arlacel mixtures but unfortunately this procedure enhances the toxicity of the bacterial cells in the same proportion. It cannot be judged from the observations so far made with the methanol extract whether the adjuvants which have been found to enhance its protective activity also increase its toxicity. There seems to be little point in investigating this aspect of the problem until progress has been made in the purification of the protective substance. It is worth recalling in this regard that much of the primary toxicity of tubercle bacilli can be traced to a cellular fraction soluble in monochlorobenzene (12). In experiments to be described in a later publication, it has been found that the killed cells of BCG, thoroughly extracted with this organic solvent, apparently retain unaltered their ability to elicit antituberculous immunity in mice. Since part of the toxic properties of killed tubercle bacilli appear to be associated with cellular components chemically distinct from those responsible for the protective activity, there is some hope that one can eventually prepare from the bacterial cells a fraction of low toxicity capable of increasing resistance against virulent infection.

SUMMARY

It is possible to prepare from tubercle bacilli a fraction soluble in methanol which is capable of eliciting in mice a marked degree of resistance against virulent tuberculous infection. The immunity was evident whether the infec-

tive dose was large and caused a disease with a rapid course, or was very small and caused a disease of many months duration.

Active material has been obtained by extraction with methanol at 55°C. of bacterial cells killed with 2 per cent phenol, and washed with acetone. The methanol extracts used in the present study have been prepared from the phenol-killed cells of a culture of BCG, and of the avirulent culture H37Ra.

Vaccination of mice has been carried out by the intraperitoneal route, and the challenge infection (with a highly virulent bovine culture), by the intravenous route.

Weight for weight, the protective activity of the methanol extract is smaller than that of the bacterial cells from which it is extracted, but its primary toxicity for mice is also considerably lower. The protective activity can be increased, and the immunity prolonged, by using certain adjuvants as vehicle for injection of the vaccine. An oil adjuvant mixture, and small amounts of a highly purified preparation of the somatic antigen of typhoid bacilli, have been found capable of enhancing and prolonging the antituberculous immunity induced by the methanol extract.

Under appropriate conditions the resistance resulting from intraperitoneal injection of the methanol extract is of the same order as that which follows vaccination with whole killed tubercle bacilli or with living BCG.

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