ANTITUBERCULOUS IMMUNITY INDUCED IN MICE BY VACCINATION WITH LIVING CULTURES OF ATTENUATED TUBERCLE BACILLI

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It has been repeatedly shown that vaccination with BCG can induce a definite, although limited, level of antituberculous immunity in experimental animals. But, though the principle of BCG vaccination is widely accepted, almost all its practical aspects have been the subject of endless controversies. These involve the stability of the BCG strain, the techniques of preparation and preservation of the vaccine, the procedures used for its administration, the extent and duration of the immunity induced, etc.

Investigation of the problems of antituberculous immunity has been rendered more difficult by the lack of simple and dependable experimental techniques for the assessment of the protective effects of vaccination. The measurement of tuberculin hypersensitivity in vaccinated individuals is a convenient technique for establishing that the vaccine has "taken," but unfortunately, it provides no information concerning the development of immunity in the vaccinated individual. Despite many assertions, there is no evidence that tuberculin allergy and immunity to infection follow parallel courses and that the former can be used as an index of the latter. Indeed, there are many reports in the literature showing that a high level of allergy can exist in the absence of immunity to infection and *vice versa.*

Guinea pigs or rabbits have of course been used extensively for the performance of protection tests which have provided fundamental information for the study of some of the phenomena of immunity. Unfortunately, vaccination increases only to a slight degree their resistance to tuberculosis, and for this reason results of statistical significance can be obtained only by the use of large numbers of animals. Because of cost, space required, and other practical limitations, these large rodents therefore hardly lend themselves to studies involving the use of multiple parameters. Yet, analysis of the development of the immune state demands that many factors be taken into consideration. The intrinsic properties of the strain used in the preparation of the vaccine, the age and other physiological characteristics of the bacilli, the dose both of vaccine and of challenge infection, the time between vaccination and infection, the

effect of the tissue environment on the response of the body to the vaccine, all these and many other factors influence greatly the results of vaccination.

In all other fields of immunological experimentation, progress has been accelerated and techniques have become more quantitative as soon as it has been possible to reproduce some of the immune reactions in a convenient and inexpensive experimental system. Since no technique is yet available for the passive transfer of antituberculous immunity or for its study *in vitro*, it is necessary at the present time to attack the problem by means of active immunization of animals.

There are on record in the literature a number of studies showing that mice can acquire a certain degree of resistance to tuberculosis following vaccination with attenuated or killed cultures of tubercle bacilli. True enough, the immunity thus obtained is of a low order, but not unlike that reported in guinea pigs or rabbits. As a complete and critical analysis of these immunization experiments has been recently presented by Swedberg (1), they need not be reviewed here. In general, immunity in mice has been measured in terms of increased survival time of the vaccinated animals over that of controls after challenge infection with virulent bacilli. The differences observed with this technique have been so slight as to require statistical analysis to give them validity. More recently, Siebenmann tried to compare by direct microscopic examination the numbers of bacilli present in the organs of infected mice (2). These results showed clearly that the multiplication of virulent bacilli was more abundant in the lungs, liver, and spleen of control mice than in the organs of animals vaccinated subcutaneously with BCG.

In the present study, an attempt was made to analyze by quantitative bacteriological techniques the effect of some of the factors which can be assumed to play a part in the immunization of mice with attenuated cultures of tubercle bacilli.

Experimental Methods

The cultures, media, and techniques used were those described in an accompanying paper (3).

RESULTS

Effect of Vaccination with Attenuated Cultures of Tubercle Bacilli on the Survival of Mice Subsequently Infected with Large Doses of Virulent Culture.—The most simple and most conclusive evidence of immunity is the ability of the vaccinated animal to survive infection with doses of virulent culture lethal for unvaccinated controls. During the early stages of the present study, several virulent cultures of human and bovine type were used in different doses for the challenge infection of the vaccinated animals. The strain MV was finally adopted in preference to the others because it is highly virulent and easy to cultivate. Doses ranging from 0.05 to 0.2 ml. of 7 to 10 day old cultures of this

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strain in tween-albumin medium were found sufficient to kill within 10 to 20 days mice 3 to 10 weeks of age; the younger the animal, the smaller the lethal dose. The survival time could be increased approximately two- or threefold by decreasing the infective dose to one-half or one-third, but the differences in survival time between vaccinated and control animals were not rendered more clean cut thereby. Although certain breeds of pigmented mice proved more susceptible than the albino animals, the latter were chosen for reasons of convenience in the present study, and large doses (usually 0.2 ml.) of culture were selected for the challenge infection in order to accelerate the test.

A first experiment was designed to test the effect of the level of atttenuation of the culture used for vaccination on the resistance of mice to virulent infection. Four cultures were compared. Three of these, R1Rv, BCG-P, and BCG-T, have been shown to be able to multiply in the mouse, but to different extents. By contrast, the fourth culture, H37Ra, seems unable to multiply *in vivo* (3). Since it has been shown in the preceding paper that the maximum population levels reached in the organs of the mouse by attenuated cultures are dependent on the route of injection, a comparison was also made of the efficacy of the intraperitoneal and intravenous routes for vaccination.

Mice were vaccinated with 0.2×10^{-4} ml. or 0.2×10^{-3} ml. of one of the four cultures used as vaccine. The smaller dose (0.2×10^{-4} ml.) was used for the two strains (R1Rv and BCG-P) shown earlier to multiply extensively in mice (3), whereas the larger dose (0.2×10^{-3} ml.) was used for both the strain BCG-T (which multiplies only little *in vivo*) and for H37Ra (which does not multiply *in vivo*). In each case, half the mice received the vaccinating dose intraperitoneally and the other half intravenously. Mice receiving only the diluent (0.1 per cent albumin) either intraperitoneally or intravenously served as controls. 2 weeks later, all the animals were infected intravenously with 0.2 ml. of virulent culture MV. The survival time (in days) for each animal is presented in Table I.

Another experiment was done to test the effect of the dose of BCG (administered intravenously) and of the length of time between vaccination and challenge infection (2 to 4 weeks) on the development of immunity against a large infective dose. Two substrains of BCG were used in comparison.

Mice were vaccinated by the intravenous route with either 0.2×10^{-2} or 0.2×10^{-4} ml. of BCG culture. The substrains of BCG used were BCG-P and BCG-T; the cultures of these two substrains were of the same age and had approximately the same optical density. Half of the animals were challenged 2 weeks after vaccination by the intravenous injection of 0.2 ml. of the virulent culture MV (grown in a medium containing human serum albumin instead of bovine serum albumin to avoid allergic reactions). The other half of the vaccinated animals received a similar virulent infection 4 weeks after vaccination. In each case, control mice having received the diluent instead of the BCG suspension were similarly infected. The survival time (in days) of animals in the different groups is given in Table II.

The results presented in Tables I and II show clearly that whatever the strain and the dose of culture used for the preparation of the vaccine, whatever

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TABLE I

Effect of Route of Vaccination and of the Attenuation of the Vaccine Strain Used on Resistance after Infection with Large Doses of Virulent Tubercle Bacilli

Vaccination			Survival time of mice infected with 0.2 ml. of virulent culture MV 2 wks. after vaccination										Average survival time
Strain	Dose	Route											
	ml.						*days*						*days*
R1Rv	0.2×10^{-4}	Intravenous	20	28	28	33	33	33	S*	S	S	S	31
"	"	Intraperitoneal	16	25	25	28	29	33	33	33			28
BCG-P	0.2×10^{-4}	Intravenous	20	22	23	25	28	33	33	S	S	S	29
"	"	Intraperitoneal	16	16	16	19	19	19	20	20	20	23	19
BCG-T	0.2×10^{-2}	Intravenous	21	22	22	26	26	30	33	33	33		27
"	"	Intraperitoneal	12	14	16	16	16	16	16	19	19	21	17
H37Ra	0.2×10^{-2}	Intravenous	19	19	19	19	20	25	25	27	33		22
"	"	Intraperitoneal	16	16	16	16	19	19	19	19	19		18
Controls Diluent	0.2	Intravenous	16	16	16	19	19	19	20	20	20	21	19
Control Diluent	"	Intraperitoneal	16	16	16	16	19	19	19	19	19	19	18

* S indicates that animals were still surviving when the experiment was terminated on the 34th day after challenge infection.

TABLE II

Effect of Vaccination on the Survival of Mice Infected with 0.2 Ml. of Virulent Culture MV

Vaccine (intravenous)		Time between vaccination and challenge infection	Survival time following challenge infection (intravenous) with 0.2 ml. MV										Average survival time
Strain	Dose												
	ml.	*days*					*days*						*days*
BCG-T	0.2×10^{-2}	15	12	12	13	17	17	17	20	21	24	25	18
"	0.2×10^{-4}	"	10	13	14	17	17	17	17	18	18	18	16
BCG-P	0.2×10^{-2}	"	10	11	11	17	17	21	24	24	24	24	18
"	0.2×10^{-4}	"	10	12	14	14	17	17	17	20	21	25	17
Diluent	0.2	"	10	10	10	10	11	12	12	13	14	14	12
BCG-T	0.2×10^{-2}	28	11	11	14	21	22	22	25	25	25	27	20
"	0.2×10^{-4}	"	11	15	15	21	21	21	21	21	22	S*	20
BCG-P	0.2×10^{-2}	"	14	15	21	21	22	25	27	27	S	S	23
"	0.2×10^{-4}	"	11	13	13	14	21	21	22	27	S	S	20
Diluent	0.2	"	11	12	13	14	15	21	21	21	21	21	17

*S indicates that animals were still surviving when the experiment was terminated on the 28th day after challenge infection.

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the route of vaccination (intravenous or intraperitoneal), and whatever the length of time elapsed between vaccination and challenge infection (2 weeks or 4 weeks), the immunity induced was never sufficient to protect the vaccinated animals against death from virulent infection. At most, it prolonged their survival time by less than twofold.

Moreover, there was in many cases overlapping of the number of days of survival time between the control and the vaccinated groups. As the groups were small (10 animals or less), some of the results have only a questionable significance. Nevertheless, a few conclusions appear justified, and have been confirmed by the results of other similar experiments not reported here.

In all cases, administration of the vaccine by the intravenous route was more effective than by the intraperitoneal route. This difference is probably related to the fact that with the doses of vaccine used, the bacilli of the vaccine multiplied much more extensively *in vivo* when they were injected intravenously rather than intraperitoneally (3). In fact, introduction of the vaccine by the intraperitoneal route resulted in a significant level of immunity only in the case of the attenuated culture R1Rv. It will be remembered that of the attenuated cultures studied, R1Rv was the one which multiplied most extensively *in vivo*, particularly following intraperitoneal inoculation (3).

Whereas R1Rv was the most effective vaccinating agent under the conditions of the test, H37Ra was the least effective. In fact, it failed to elicit any significant immunity whether injected by the intraperitoneal or by the intravenous route, and this, despite the fact that the amount injected (0.2×10^{-3} ml.) was ten times larger than the amount used in the case of cultures R1Rv and BCG-P. The failure of H37Ra to elicit immunity under the conditions of the test is certainly a reflection of the fact that this strain is not capable of multiplying *in vivo* (3).

The two BCG substrains tested (BCG-P and BCG-T) were more effective than H37Ra, but less effective than R1Rv (Table I), a finding in agreement with the fact that they multiply *in vivo* less extensively than the latter culture.

Multiplication of Virulent Tubercle Bacilli in the Spleen and Lungs of Mice Vaccinated with BCG.—As pointed out in a preceding paragraph, it is difficult to assess the effect of vaccination with attenuated cultures from a comparison of the length of survival time of vaccinated animals with that of the controls. The differences observed were always small, and there was often much overlapping, thus rendering the results inconclusive in many cases. Experiments were therefore instituted to determine whether immunity could be assessed by another technique, namely in terms of its effect on the multiplication of small doses of virulent bacilli in the organs of mice.

Several independent variables were introduced in the following experiment. Two different substrains of BCG were used, different doses of vaccine, different periods of time between vaccination and challenge infection. The data

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collected included the number of BCG bacilli in the organs of mice at the time of the challenge virulent infection, and the number of virulent bacilli that could be recovered from the spleen and lungs of mice sacrificed 2 weeks after virulent infection.

Mice were vaccinated by the intravenous route with either one of the two BCG substrains (BCG-P and BCG-T). In the case of each substrain, three doses of vaccine were used: 0.2 10^{-3}, 0.2×10^{-4}, and 0.2×10^{-5} ml. for BCG-P, and 0.2×10^{-2}, 0.2×10^{-3}, and 0.2×10^{-4} ml. for BCG-T. Other mice receiving only the diluent served as unvaccinated controls. Three mice of each group were sacrificed 2, 3, 5, 7, 10, 14, and 17 weeks after vaccination. The number of BCG bacilli in the spleen and lungs was determined by the usual technique.

On the same day, four other mice of each group were infected with 0.2×10^{-5} ml. of the virulent culture MV. 2 weeks later, these mice were sacrificed and the number of living bacilli in their spleen and lungs determined. For the sake of brevity, only the data pertaining to the 2 week, 7 week, and 14 week periods are presented in Table III. Fig. 1 gives in a diagrammatic form the trend of the bacterial populations in the spleens of the different groups of mice over the whole period of observation (17 weeks). Results are plotted for the 0.2×10^{-3} ml. dose of vaccine.

In the case of mice vaccinated with BCG-T and challenged with virulent bacilli MV, differences in colonial morphology made it possible to distinguish on the agar plates the colonies of BCG from those of the virulent strain. The figures reported in Table III for this group of mice (and the values used for preparing Fig. 1) refer only to the colonies of virulent bacilli.

In the case of mice vaccinated with BCG-P and challenged with the virulent MV bacilli, it was not judged possible to differentiate between the two types of colonies with certainty. Hence the figures for the numbers of colonies recovered from mice vaccinated with BCG-P and challenged with MV include the two types of colonies. The numbers of BCG colonies recovered from mice not infected with MV are indicated in the left portion of Table III.

The results presented in Table III and Fig. 1 reveal that virulent bacilli had multiplied much more abundantly in the control mice than in the mice which had been vaccinated with 0.2×10^{-2} or 0.2×10^{-3} ml. of vaccine 2 weeks before challenge infection. The difference between control groups and the groups vaccinated with BCG-P appears particularly striking when it is remembered that the colonies recovered from the latter include not only the virulent bacilli, but also the BCG bacilli used for vaccination (figures in the left columns of Table III).

It took longer, (5 to 7 weeks) for immunity to become manifest in the groups of mice which received smaller doses of vaccine. With the larger doses it was not possible to detect any difference between the levels of immunity induced by the two BCG substrains. But it will be noted that 0.2×10^{-4} ml. of BCG-T was less effective than the same dose or even than 0.2×10^{-5} ml. of BCG-P. The differences between the two became apparent when the challenge infection was given 10 weeks or later after vaccination. This finding is to be related to the fact that, in agreement with the results of earlier experiments, BCG-P multiplied more extensively in the mouse than did BCG-T (3).

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Duration of Immunity Induced in Mice by Vaccination with BCG.—In the preceding experiment, it was found that mice infected with virulent bacilli

TABLE III

Effect of Strain of BCG, Dose, and Time after Vaccination on the Level of Antituberculous Immunity

Strain	Dose	Time between vaccination and challenge infection	No. ($\times 10^{-2}$) of colonies of vaccinating BCG bacilli in mice at time of challenge infection						No. ($\times 10^{-2}$) of colonies recovered from mice 2 wks. after challenge infection with 0.2 $\times 10^{-5}$ ml. virulent MV					
			Spleen			Lungs			Spleen			Lungs		
	ml.													
BCG-T	0.2 $\times 10^{-2}$	2 wks.	56‡	52	50	10‡	9		90	70	42	26	22	—
	(56000)*	7 "	6	2	2	4	2	0	480	110	88	20	2	0
		14 "	0	0	0	0	0	0	460	390	230	16	10	—
BCG-T	0.2 $\times 10^{-3}$	2 wks.	18	12	—	2	0	0	370	380	310	30	24	22
	(5600)	7 "	0	0	0	0	0	0	480	—	—	6	2	—
		14 "	—	—	—	—	—	—	520	380	—	4	4	—
BCG-T	0.2 $\times 10^{-4}$	2 wks.	0	0	0	0	0	0	3,200	2,400	600	680	240	—
	(560)	7 "	0	0	0	4	0	0	660	650	300	280	16	12
		14 "	—	—	—	—	—	—	1,150	390	—	18	0	0
BCG-P	0.2 $\times 10^{-3}$	2 wks.	700	340	66	30	12	6	420	190	82	260	210	126
	(6400)	7 "	190	36	30	34	32	20	76	52	14	60	28	12
		14 "	12	10		6	6	—	42	18	0	0	0	0
BCG-P	0.2 $\times 10^{-4}$	2 wks.	70	50	46	12	8	4	4,100	3,400	480	310	30	20
	(640)	7 "	48	28	22	64	12	—	108	106	—	4	4	2
		14 "	4	2	2	0	0	—	48	26	—	4	—	—
BCG-P	0.2 $\times 10^{-5}$	2 wks.	8	8	8	6	0	—	4,910	5,400	3,500	450	390	38
	(64)	7 "	130	22	12	0	0	0	1,240	430	66	18	2	2
		14 "	0	0	0	0	0	0	132	74	34	16	0	0
Control														
Diluent	0.2	2 wks.							13,500	13,100	12,400	6,000	5,500	4,800
		7 "							4,300	4,300	3,800	610	370	26
		14 "							6,000	4,500	3,900	222	50	0

In the mice challenged after vaccination with BCG-T the figures represent the numbers of virulent bacilli (MV) recovered, whereas in the groups challenged after vaccination with BCG-P, the figures represent the total of virulent bacilli (MV) and vaccinating bacilli (BCG-P). See text for explanation.

—, the count was unavailable for technical reasons.

* Figures in parentheses indicate numbers of viable bacillary units (single cells or clumps) present in the volume of vaccine used (as determined by plate counts on albumin agar).

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

as late as 17 weeks after vaccination with BCG still displayed marked antituberculous immunity (see Fig. 1); results for the 17 week period have been omitted from Table III. The duration of the immunity is illustrated by another technique in the following experiment.

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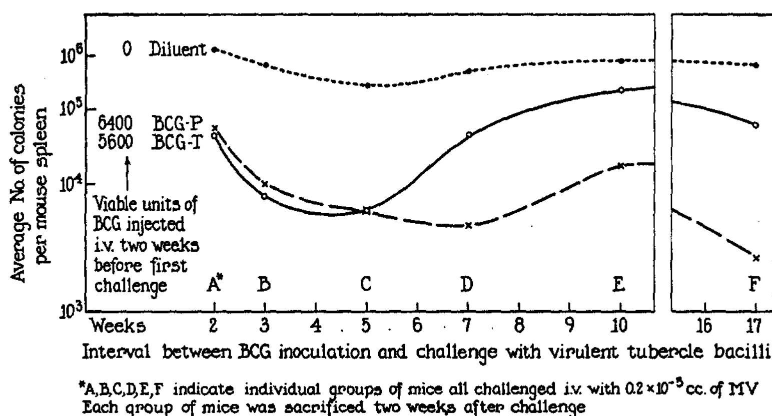

FIG. 1. Recovery of virulent tubercle bacilli (MV) from mice infected at various intervals after vaccination with living BCG-T or BCG-P. The figures plotted on the curves represent the average number of colonies obtained per spleen from groups of 3 mice. The animals were challenged by intravenous injection of 0.2×10^{-5} ml. of MV at the times indicated in the chart and were sacrificed 2 weeks later.

TABLE IV

Duration of Immunity Induced by Intraperitoneal Vaccination with 0.2 × 10^{-2} Ml. of Living BCG-P

Time after infection with 0.2×10^{-5} ml. MV	No. ($\times 10^{-2}$) of colonies recovered from spleen or lungs of															
	Unvaccinated mice								Mice challenged 2 wks. after vaccination							
	Spleen				Lungs				Spleen				Lungs			
days																
1	0	0	0	0	0	0	1	0	210*	170	96	9	310	4	0	—
8	6	0	0	0	0	2	2	0	155	138	99	71	18	13	10	1
14	1,070	560	220	—	0	11	0	0	86	84	28	—	35	7	6	—
32	1,400	960	570	200	500	440	420	—	160	72	61	34	76	63	1	—
56	500	260	220	190	1900	740	560	0	57	50	38	30	32	30	30	21

In the mice challenged after vaccination with BCG-P, the figures represent the total of virulent bacilli (MV) and vaccinating bacilli (BCG-P). See text for explanation.

—, the count was unavailable for technical reasons.

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

Mice were infected by the intraperitoneal route with 0.2×10^{-2} ml. of culture BCG-P. The unvaccinated controls received 0.2 ml. of diluent instead. 2 weeks later, all animals were infected by the intravenous route with 0.2×10^{-5} ml. of the virulent culture MV. Three controls and three vaccinated animals were sacrificed after 1, 8, 14, 32, and 56 days. The numbers of living bacilli in the spleen and in the lungs were determined by the usual techniques (Table IV).

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In confirmation of the results of earlier experiments (3), the numbers of living virulent bacilli in the spleens of the control animals reached a maximum some 2 weeks after infection, and then remained more or less stationary. By contrast, the numbers of bacilli in the lung did not become large until after the 4th week.

In the vaccinated animals appreciable numbers of bacilli could be recovered from the spleens and lungs the very first day after the challenge infection, but these bacilli evidently were the BCG organisms that had been used for vaccination (the vaccinating dose was large, 0.2×10^{-2} ml.) since there were no virulent MV colonies recovered at this period from mice which had not been vaccinated. The numbers of bacterial colonies recovered from the vaccinated animals did not increase at any time throughout the period of observation; even though the bacterial population included both BCG and the virulent bacilli, it was still far smaller than in the non-vaccinated mice 56 days after infection.

Effect of Treatment with Isoniazid on the Development of Immunity in Mice Vaccinated with the Attenuated Culture R1Rv.—If it be true as appears from the results presented in this report that the antituberculous immunity induced by attenuated living cultures is an expression of the extent of multiplication of these cultures in the vaccinated animals, it should be possible to interfere with the development of immunity by treating the animals with a suitable antibacterial drug shortly after vaccination. Isonicotinic acid hydrazide (isoniazid) is such a substance. Using it, this hypothesis has been put to test in the following experiments.

On April 22, 1952, 48 mice received by the intraperitoneal route 0.2×10^{-2} ml. of the attenuated culture R1Rv; 16 animals received the diluent instead. On April 23, 1952, *i.e.* on the day after vaccination, 16 of the vaccinated mice received by the intraperitoneal route 1 mg. (in 0.5 ml.) of isonicotinic acid hydrazide (isoniazid). Therapy was continued until May 9, 1952, *i.e.* for 18 days, by supplying the drug in the diet at a dosage of approximately 1.0 mg. daily (the diet had the following composition: desiccated skimmed milk, 330 gm.; white wheat flour, 660 gm.; salt mixture, 10 gm.; isoniazid, 0.5 gm. added in solution in 1000 ml. of 15 per cent gelatin).

On April 28, 1952, *i.e.* 6 days after vaccination, another group of 16 vaccinated mice was also started on isoniazid diet, which was also continued until May 9, 1952, *i.e.* for 12 days. The third group of 16 mice was placed on isoniazid diet on May 2, 1952, *i.e.* 11 days after vaccination, and continued until May 9, 1952, (7 days of therapy). Half of the non-vaccinated mice received the drug from May 2, 1952, to May 9, 1952. The animals of each of the groups (vaccinated and controls) were divided into four subgroups. In each case, a first subgroup was infected by the intravenous route with 0.2×10^{-5} ml. of virulent culture, on May 12, 1952; a second, on May 19, 1952; a third, on May 26, 1952; and the fourth on June 2, 1952. In other words, the challenge infection for each of the four subgroups was given 3, 4, 5, and 6 weeks after vaccination; *i.e.*, 3, 10, 17, and 24 days after cessation of isoniazid therapy.

The mice were sacrificed 2 weeks after the challenge infection. The numbers of bacterial colonies recovered from their spleens and lungs are given in Table V.

Similar groups and subgroups received the vaccinating dose and the isoniazid therapy under the same conditions and at the same time as the ones described above. However, these

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mice were not infected with virulent bacilli. The animals of each subgroup were sacrificed at the same time when those of the duplicate subgroup received the challenge infection. This permitted the determination of the number of R1Rv bacilli in the spleen and lungs of the vaccinated animals at the time of the challenge infection. These numbers are also presented in Table V (in parentheses).

TABLE V

Effect of Treatment with Isoniazid on Development of Immunity in Mice Vaccinated with 0.2 × 10⁻⁵ Ml. R1Rv

Group	Date of vaccination with R1Rv	Dates of treatment with isoniazid	No. (× 10⁻²) of bacterial colonies recovered from mice sacrificed 2 wks. after having been challenged with virulent MV at following times after vaccination							
			3 wks.		4 wks.		5 wks.		6 wks.	
			Spleen	Lungs	Spleen	Lungs	Spleen	Lungs	Spleen	Lungs
I	4/22/52	4/23/52 to 5/9/52	4,266* (0)‡	1,656 (0)	5,352 (0)	8,932 (0)	1,514 (0)	274 (2)	1,910 (0)	530 (18)
II	4/22/52	4/28/52 to 5/9/52	2,010 (0)	1,166 (0)	1,900 (0)	2,210 (0)	712 (0)	340 (0)	118 (32)	60 (6)
III	4/22/52	5/2/52 to 5/9/52	806 (4)	210 (0)	606 (4)	— (0)	190 (72)	8 (2)	60 (412)	92 (76)
IV	No vaccination	5/2/52 to 5/9/52	1,850	960	3,360	2,720	1,732	1,776	1,866	1,160
V	No vaccination	No treatment	3,684	1,304	4,750	6,116	3,100	2,810	1,350	882

—, the count was unavailable for technical reasons.

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

‡ The figures in parentheses represent the numbers of colonies of R1Rv recovered from a duplicate set of mice which had been vaccinated but not challenged and were sacrificed at the time of challenge infection of the other set.

It is obvious, (Table V) that the drug isoniazid exerted a marked antibacterial effect on R1Rv. No data are available in the case of this particular experiment concerning the multiplication of the R1Rv bacilli in the mice not receiving the isoniazid therapy. However, it is known from earlier experiments that the numbers in the spleen and lungs 3 to 6 weeks after vaccination were certainly large. By contrast, hardly any R1Rv bacilli (figures in parentheses in Table V) could be recovered at first from the groups of mice treated with the drug. But 2 weeks after cessation of therapy, the R1Rv bacilli began to appear, first in the group of mice which had remained without therapy for the first

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10 days after vaccination (group III), then in the group in which the therapy had been delayed only 6 days (group II). Thus, although therapy begun shortly after injection of the R1Rv bacilli and continued for several days had greatly retarded bacterial multiplication, it had not been able to completely eradicate the bacilli from the tissues, since they began to multiply again after withdrawal of the drug. This complex situation is reflected in the development of immunity.

When mice were challenged with virulent infection on May 12, 1952, *i.e.* 3 days after withdrawal of the drug, only those of group III exhibited some evidence of immunity. It seems certain that during the first 12 days without therapy (April 22, 1952 to May 2, 1952) sufficient multiplication of R1Rv had taken place to initiate the immunization process in this group.

In mice challenged 5 weeks after vaccination, immunity had become very striking in group III and was becoming manifest in group II. Although R1Rv bacilli could not be recovered from mice of group II at the time that these received the virulent culture MV it is apparent that multiplication of the vaccine was then beginning in these animals, as indicated by the fact that the R1Rv bacilli could be readily recovered from the lungs and spleen of mice of the same group sacrificed 1 week later.

Immunity was fully evident in mice of groups II and III infected with virulent bacilli 6 weeks after vaccination. However, it had not yet appeared in group I which had been treated with isoniazid immediately after vaccination, and in which the R1Rv bacilli had not had time to multiply sufficiently at the time of the challenge infection to elicit an immune response.

DISCUSSION

Mice vaccinated with living tubercle bacilli of the "attenuated" type rapidly developed partial resistance against infection with virulent bacilli. Under the conditions of the experiments described in the present paper the numbers of living virulent bacilli that could be recovered from the spleen of the vaccinated animals 2 weeks after infection were some ten times smaller than the numbers recovered from the organs of the control mice. The data presented also suggest that the spread among the numbers of bacilli in the lungs of the vaccinated and non-vaccinated animals became greater with time as the infection progressed. This conclusion will be documented more fully in reports to follow.

The immunity induced by vaccination was directly related to the extent of multiplication of the attenuated culture in the tissues of the vaccinated animal, as indicated by the following facts.

(*a*) No immunity could be detected following vaccination with cultures of the "attenuated" strain H37Ra, which had been found unable to multiply in the mouse.[1]

[1] Seemingly this statement is in contradiction with the results presented in the following paper (4). It will be noticed however, that antituberculous immunity could be produced with

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(*b*) Small doses of culture of attenuated strains proved far more effective in eliciting immunity when injected by the intravenous route than by the intraperitoneal route, a finding which falls in with the fact that the numbers of bacilli recovered from the spleen and lungs of mice were greater when the culture was injected intravenously. In unpublished experiments it has been found that the subcutaneous and intramuscular routes were notably ineffective for infecting mice with attenuated bacilli. Similarly, these routes proved very ineffective for the production of immunity. It is true that other workers in the past have obtained definite evidence of immunity in mice vaccinated by the subcutaneous route (1, 2), but the amounts of vaccine that they used were very large, of the order of a thousandfold those used in the present study.

(*c*) When very small doses of vaccine were used, of the order of 10^{-5} ml., it took several weeks for the immune state to become established. This period corresponded to that required for the establishment of a sufficient population level of the attenuated culture in the vaccinated animal.

(*d*) The less attenuated the culture used as vaccine, *i.e.* the higher the population level which it reached in the mouse, the more effective it was as a vaccinating agent. This conclusion had been reached by Trudeau some 50 years ago on the basis of immunization experiments in guinea pigs and rabbits (8), with strains (other than BCG) of various degrees of virulence, and it has since been confirmed by other workers (1, 9, 10).

By the proper manipulation of variables (dose of vaccine, route of vaccination, time after vaccination) it was possible to demonstrate differences between the two substrains of BCG tested; the substrain which proved the more effective in eliciting immunity being also the one which multiplied more extensively *in vivo*.

(*e*) The development of the immune state following vaccination could be retarded or completely prevented by treatment of the mice in course of immunization with isonicotinic acid hydrazide.

By the use of the proper experimental technique, it was possible without fail to demonstrate that vaccination with cultures of attenuated bacilli induced in mice a state of increased resistance to virulent tubercle bacilli. But the immune mechanism was only partially effective. In no case did it result in death of the virulent bacilli; it only retarded or at best interrupted their multiplication. In no case were the vaccinated animals protected against death from infection with large doses; they only survived somewhat longer than the

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killed H37Ra bacilli, only when the amount of bacillary material injected was very much larger (a 1000-fold) than the amount of *living* bacterial culture used in the present study. Authors who have been successful in obtaining antituberculous immunity by vaccinating guinea pigs with living cultures of H37Ra (5, 6, 7) used amounts of culture much in excess of those used in the present study, so large indeed that no multiplication of H37Ra was required to produce an adequate antigenic stimulus.

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non-vaccinated controls. Limited though it was, the immunity persisted for many weeks, but it began to fade shortly after the bacilli used as vaccine had disappeared from the tissues.

Granting all these limitations, it nevertheless appears profitable to use the mouse for the study of many problems of antituberculous immunity. Young mice, in particular, appear to lend themselves well to tests for the quantitative assessment of vaccines and of vaccination procedures. Since all evidence—experimental as well as epidemiological—points to the conclusion that immunity to tuberculosis does not operate through a bactericidal action on the infecting bacilli, but merely or chiefly by restricting their multiplication, it seems certain that analysis of the immune mechanisms requires the use of techniques permitting a quantitative appraisal of the number of viable bacilli in the tissues. In this relation the bacteriological procedures used in the present study would seem to constitute a useful adjunct to histological techniques.

SUMMARY

The immunity induced in mice by vaccination with living attenuated cultures of tubercle bacilli was measured by two criteria.

(*a*) Increase in survival time of the vaccinated animals after infection with a dose of virulent bacilli sufficient to kill all the unvaccinated controls within 10 to 20 days.

(*b*) Difference in the number of living bacilli recovered from the spleen and lungs of vaccinated and normal animals infected with a small dose of virulent bacilli.

The level of immunity induced was found to depend upon the extent of multiplication *in vivo* of the bacilli used for vaccination. This in turn was conditioned by the degree of attenuation characteristic of the bacterial strain used in the preparation of the vaccine, the amount of vaccine injected, the route of vaccination, and the time interval between vaccination and challenge infection. It was possible to prevent or retard the development of immunity by treating the mice in course of immunization with a drug, isoniazid, capable of interrupting the multiplication *in vivo* of the bacilli used as vaccine.

Although immunity regularly developed and lasted for many weeks when the proper conditions of vaccination were used, the immune response was never sufficient to protect the animals against ultimate death from infection with virulent tubercle bacilli. The prolongation of life in the vaccinated mice was not consequent on a direct bactericidal effect but rather on a retarded or interrupted multiplication of the virulent bacilli *in vivo*.

The quantitative bacteriological techniques used in the present study would appear to be of value for the analysis of certain problems of immunity, and for the appraisal of vaccines and techniques of vaccination.

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BIBLIOGRAPHY

1. Swedberg, B., *Acta med. scand.*, 1951, **139,** suppl. 250, 1.
2. Siebenmann, C. O., *J. Immunol.*, 1951, **67,** 137.
3. Pierce, C. H., Dubos, R. J., and Schaefer, W. B., *J. Exp. Med.*, 1953, **97,** 189.
4. Dubos, R. J., Schaefer, W. B., and Pierce, C. H., *J. Exp. Med.*, 1953, **97,** 221.
5. Steenken, W. J., and Gardner, L. U., *Yale J. Biol. and Med.*, 1943, **15,** 393.
6. Steiner, M., and Zuger, B., *J. Immunol.*, 1943, **46,** 83.
7. Zuger, B., and Steiner, M., *J. Immunol.*, 1943, **46,** 91.
8. Trudeau, E. L., *Tr. Nat. Assn. Study and Prev. Tuberc.*, 1905, 157.
9. Lange, B., and Lydtin, K., *Z. Hyg. u. Infektionskrankh.*, 1929, **110,** 209.
10. Yanagisawa, K., Kanai, K., Shiga, Y., and Ito, F., *Jap. Med. J.*, 1951, **4,** 1.

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