

BEHAVIOR OF ATTENUATED MYCOBACTERIA IN ORGANS OF NEONATAL AND ADULT MICE*†

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As reported in earlier papers Calmette-Guérin bacillus (BCG)¹ administered by the peritoneal route persists in organs of adult mice in a condition of bacterial stasis from the outset of its injection (1). Recent experiments have revealed, however, that the in vivo behavior of BCG is entirely different when it is administered to mice shortly after their birth. In newborn animals, extensive bacterial multiplication occurs in both splenic and pulmonary tissue. Recognition of this difference in the behavior of BCG in newborn and in adult animals has given a better understanding of the effect of in vivo vaccinal multiplication in the development of immune resistance. The experiments to be described confirm earlier findings by showing that vaccinal multiplication *per se* is not an absolute requirement for the development of antitubercular immunity.

Materials and Methods

Animals.—The materials and methods used in this study have been detailed previously (1, 2).

Mice of the NCS strain were maintained on a commercial pellet diet (Dietrich & Gambrell Inc., Frederick, Md.). Animals were obtained at different times after their birth. Except where noted, only female animals were used.

Vaccination.—Lyophilized ampules of the Montreal strain of BCG vaccine (Lot No. 1364-4, generously supplied by Dr. A. Frappier of the Institute of Hygiene of Montreal) were reconstituted with albumin-water immediately before use. Dilutions of vaccine were made in albumin-water. All injections were by the peritoneal route; adult mice received 0.2 ml inoculum and newborn mice 0.025 or 0.05 ml. The technique used for peritoneal injection of newborn mice is described in reference 2.

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¹ *Abbreviation used in this paper:* BCG, Calmette-Guérin bacillus.

Challenge.—The H37Rv strain of virulent mycobacteria was used. Respiratory challenge infection was performed in a Middlebrook-type aerosol chamber (Tri-R Instruments, Inc., Rockville Centre, N.Y.). Infective aerosols were obtained by nebulizing 10 ml of suspension containing approximately 10^6 viable units of mycobacteria/ml over a 60 min period.

Enumeration of Organisms.—Mice were sacrificed at various intervals of time after vaccination or challenge and their lungs and spleens homogenized in albumin-water in Teflon glass tissue grinders. Samples of homogenate were diluted in albumin-water and suitable portions were pipetted into sterile screw cap tubes to which was added 2 ml of soft agar medium (3). The number of viable mycobacteria was estimated from the number of colonies which developed within the media. Challenge organisms were distinguished from BCG bacilli by use of the differential inhibitor 2-thiophenecarboxylic acid hydrazide. The inhibitor, in water, was sterilized by filtration and added to the soft agar medium in a final concentration of 15 μ g/ml.

RESULTS

Behavior of Peritoneally Injected Mycobacteria in Adult Mice.—As previously described, BCG fails to multiply in the animals' organs when injected into adult mice by the peritoneal route (1). This tuberculostatic effect is illustrated in the following experiment.

6-wk old female NCS mice were peritoneally injected with $10^{4.6}$ viable units of Montreal strain BCG. For comparative purposes, a group of 8-wk old female animals was given $10^{4.3}$ viable units of virulent human bacilli (H37Rv) by intraperitoneal injection. 1 day later, and at intervals thereafter, groups of five mice were sacrificed and the number of mycobacteria present in their lungs and spleens was ascertained. The results are given in Table I.

BCG organisms appeared in both splenic and pulmonary tissue 24 hr after infection (Table I). Similar numbers of bacilli were recovered from the infected animals' spleens at this time and at all intervals over the following 10 wk. Neither extensive multiplication nor destruction of vaccine organisms occurred. The vaccine bacilli also failed to multiply in the animals' lungs. A progressive decline of the mycobacterial population occurred in this organ, however, and 10 wk after vaccination few bacilli were obtained from the animals' pulmonary tissues.

Peritoneally injected virulent mycobacteria behaved in an entirely different fashion. The initial uptake of human bacilli by the animals' organs was much the same as that of BCG. However, progressive multiplication of virulent organisms occurred in both lung and spleen tissue. Rapid growth occurred in both organs for the first 2 wk after challenge; thereafter the infection declined and persisted in a relatively constant "steady" state.

Comparative Behavior of BCG in Newborn and Adult Mice.—The growth of peritoneally injected BCG in organs of mice of different ages was next studied.

Newborn mice received 0.05 ml of vaccine containing either $10^{4.2}$ or $10^{6.2}$ viable units of BCG. The vaccinated animals were then intermixed and reapportioned, in groups of eight mice, to randomly selected untreated foster mothers. 21 days later the infected mice were weaned and recaged in groups of five. Groups of 5-wk old and 20-wk old female mice had

been simultaneously injected with 0.2 ml of vaccine containing $10^{4.2}$ or $10^{6.2}$ viable units of BCG.

1 day after administration of BCG, and at intervals thereafter, one vaccinated mouse from each of five nursing litters and five animals of each of the older groups were killed and the number of BCG organisms present in their spleens and lungs determined. The results of this experiment are graphically illustrated in Figs. 1 A and 1 B.

TABLE I
Mycobacteria Recovered from Organs of Mice at Various Times after Their Peritoneal Administration

Organism injected*	Time after injection	No. of colonies (log) obtained from organ†	
		Spleen	Lung
BCG	1 day	3.1 ± 1.0	1.7 ± 0.5
	1 wk	3.7 ± 0.7	1.9 ± 0.1
	2 wk	2.9 ± 1.2	0.2 (2/5)
	4 wk	2.8 ± 0.7	0.5 (2/5)
	6 wk	2.6 ± 0.4	0.4 (2/5)
	10 wk	2.6 ± 0.7	0.0 (0/5)
H37Rv	1 day	2.7 ± 0.3	1.0 ± 0.2
	1 wk	4.6 ± 0.6	2.2 ± 0.7
	2 wk	6.0 ± 0.6	3.3 ± 0.6
	4 wk	5.0 ± 0.1	3.3 ± 0.8
	12 wk	5.0 ± 0.1	3.3 ± 0.8

* Animals were peritoneally injected with $10^{4.6}$ viable units of BCG or with $10^{4.3}$ viable units of H37Rv.

† Number of colonies of BCG or H37Rv organisms (expressed in logs) obtained from tissue at given time after infection ± standard deviation. Values in parenthesis refer to number of organs positive out of total number tested. Otherwise values are arithmetical average for five organs. All values are for 1 ml of tissue homogenate (out of a total of 5 ml).

Fig. 1 A illustrates the fate of BCG in animals given the heaviest dose of vaccine. BCG organisms appeared in both splenic and pulmonary tissue of newborn animals immediately after infection. The vaccine organisms multiplied extensively in both organs for the first 6 wk after infection. Between the 6th and 10th wk, however, the numbers of colonies obtained from spleens declined slightly and then became stable. The number of colonies obtained from pulmonary tissue steadily declined after the 6th wk.

The fate of BCG organisms in the spleens and lungs of 5- and 20-wk old animals was similar to that described in the first experiment. Organisms were immediately taken up by spleens and lungs of adult mice. Extensive multiplication of the vaccine, however, did not occur in either splenic or pulmonary

tissue. The number of colonies recovered from pulmonary tissue progressively declined while the number from splenic tissue persisted without significant change.

Similar results were obtained with mice given the smaller dose of BCG (Fig. 1 B). Very few organisms were recovered from the organs of newborn mice immediately after infection. Yet extensive multiplication occurred in these

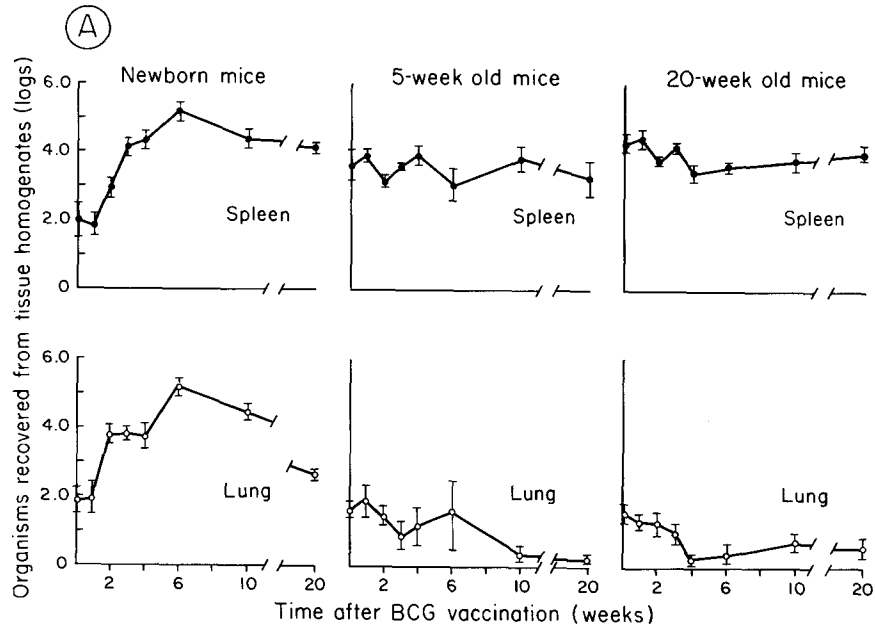


FIG. 1 A. Growth of BCG in organs of mice peritoneally injected with BCG at given age. Average of five animals \pm SD. Each animal received 1.5×10^6 vaccine organisms.

mice and large numbers of bacilli were recovered from their pulmonary and splenic tissue 6 wk after infection.

Larger numbers of vaccine organisms were taken up by organs of adult mice, but there was no evidence of bacillary multiplication in either spleen or lung tissue. It is worth noting that although a greater number of organisms were initially recovered from the organs of animals vaccinated as adults, the number of BCG colonies obtained 6 wk after infection was much smaller than was obtained from animals vaccinated at birth.

Age of Animals and In Vivo Multiplication of BCG.—The age at which splenic and pulmonary tissue became resistant to the multiplication of peritoneally injected BCG was ascertained in the following experiment.

Animals of several ages were peritoneally injected with 1.5×10^6 viable units of BCG. The infection was administered in various volumes of inocula related to the size of the animal. 1-day old animals received 0.05 ml of vaccine suspension containing 6×10^6 viable units of BCG. 1- and 2-wk old mice were given 0.1 ml of this suspension diluted in half. 3-wk and older mice were injected with 0.2 ml of the vaccine diluted fourfold. On the day after administration of vaccine, five representative animals in each group were sacrificed and the number of BCG present in their spleens and lungs determined. Similar determinations were made at intervals thereafter. The height of the vaccine infection was found to occur, in all cases save one, at the 6th wk after administration of the vaccine. The results are described in Tables II and III.

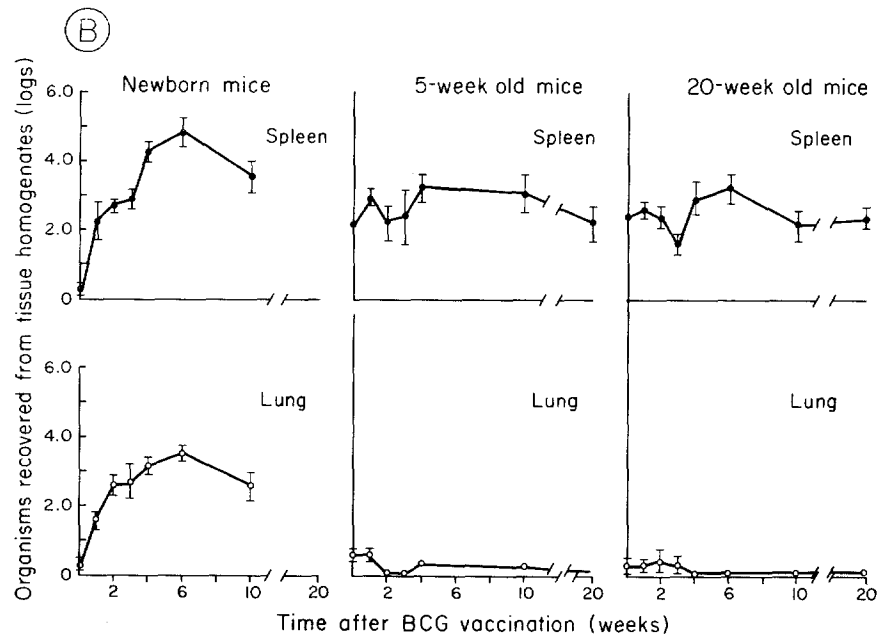


FIG. 1 B. Growth of BCG in organs of mice peritoneally injected with 1.5×10^4 vaccine organisms.

For simplicity of illustration, only the numbers of organisms recovered 1 day and 6 wk after challenge are presented.

As seen in Table II, both spleens and lungs of animals vaccinated at birth supported extensive multiplication of vaccine organisms. Significant growth of BCG also occurred in lungs of mice vaccinated when they were 1 wk old, but multiplication was less extensive in their spleens. When mice were 2 wk of age or older at the time of vaccination, their spleens and lungs were largely able to restrain the multiplication of vaccine bacilli.

A significant difference was again found in the number of organisms initially taken up by splenic tissue of animals of different ages. The initial uptake of

TABLE II
Multiplication of BCG in Organs of Mice Injected by the Peritoneal Route at Different Ages

Age at infection*	No. of colonies obtained from organs at given time after infection† (logs)					
	Spleen			Lung		
	1 day	6 wk	Multiplicity‡	1 day	6 wk	Multiplicity‡
1 day	1.6 ± 0.6	5.2 ± 0.5	3.2	1.9 ± 0.7	5.1 ± 0.4	2.7
1 wk	3.1 ± 0.5	4.3 ± 1.2	1.4	2.3 ± 0.5	4.8 ± 0.7	2.1
2 wk	3.8 ± 1.1	4.5 ± 0.8	1.2	2.7 ± 0.9	3.7 ± 1.2	1.4
3 wk	4.4 ± 0.5	3.2 ± 0.6	-0.7	2.0 ± 1.8	2.8 ± 1.7	1.4
5 wk	3.6 ± 1.2	3.0 ± 1.4¶	-0.8	1.6 ± 0.4	2.3 ± 1.4¶	1.4
20 wk	4.3 ± 0.5	3.7 ± 0.6	-0.9	1.3 ± 0.8	0.9 ± 0.7	-0.7

* Animals peritoneally injected with Montreal strain BCG. 1-day old animals received 0.05 ml; 1- and 2-wk old animals, 0.1 ml; remaining ages, 0.2 ml volumes of the inoculum diluted to contain $10^{6.2}$ viable units of BCG in each case.

† Figures refer to number of BCG colonies (expressed in logs) obtained from 1 ml (out of 5) of organ homogenate of animals at given times after peritoneal infection ± standard deviation. Also see Table I.

‡ Multiplicity = log No. of colonies obtained 6 wk after vaccination divided by log No. of colonies obtained 1 day after vaccination.

|| Indicates values significantly different ($P = <0.05$) from those of comparable 1 day value.

¶ Time of sacrifice was 5 wk instead of 6 wk.

TABLE III
*Uptake and Growth of BCG in Lungs of Mice Injected by the Peritoneal Route at Different Ages**

Age of injection	BCG recovered from pulmonary tissue 1 day and 6 wk after infection†		
	1 day	6 wk	Approximate multiplication
1 day	350	940,000	2500
1 wk	1000	257,000	250
2 wk	3310	16,000	5
3 wk	2800	800	—
5 wk	420	1,000‡	2
20 wk	220	50	—

* See Table II.

† Average total number of BCG organisms obtained from entire lungs of five animals.

‡ Time of sacrifice was 5 wk instead of 6 wk.

BCG by pulmonary tissue, however, was much the same irrespective of the animals age. The results described in Table II for pulmonary tissue are therefore reillustrated in Table III which gives the total number of colonies obtained rather than logarithmic values. As may be seen, relatively few bacilli were taken up by pulmonary tissue of any animal after vaccine injection. Appre-

ciable multiplication occurred, however, in pulmonary tissue of 1-day and 1-wk old animals. Little or no multiplication occurred in the lungs of older animals.

Effect of Age at Vaccination on the Development of Immunity.—The resistance attained by animals vaccinated at different ages against superinfection with virulent bacilli was assessed by airborne challenge infection. The technique used for this protection test has been previously described (2). In brief, it is based on the fact that vaccinated animals show a limited but significant inhibition of

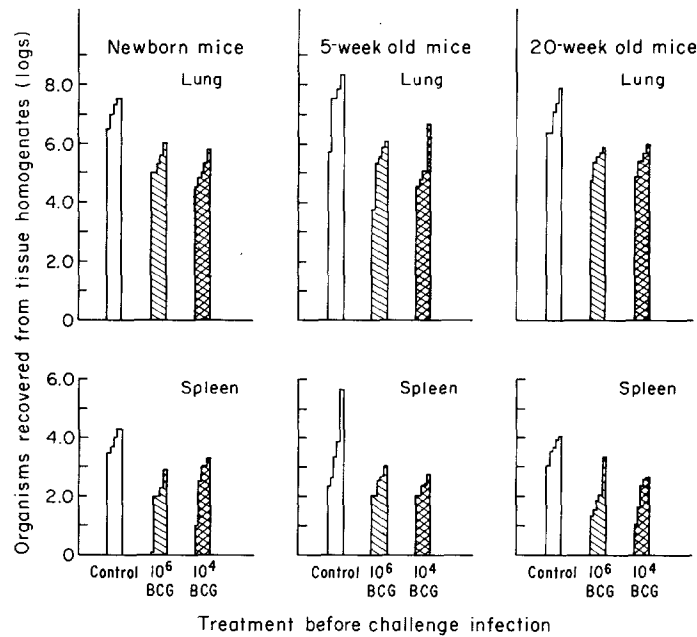


FIG. 2. Number of virulent human bacilli in organs of control and vaccinated mice 4 wk after aerosol challenge infection. Animals were peritoneally vaccinated with BCG at given ages, 10 wk before challenge. Bars refer to colonies obtained from five individual animals.

challenge bacilli in both pulmonary and splenic tissue between the 2nd and 6th wk after airborne infection. Retardation of mycobacterial growth is most obvious 4 wk after challenge infection when the virulent bacilli recovered from organs of vaccinated animals are 1–2 logs less numerous than in control animals.

Mice were vaccinated when newly born or when 5 or 20 wk of age, as described in the preceding section. 10 wk after vaccination these animals, along with comparable untreated animals, were challenged with virulent human bacilli. For this purpose the animals were exposed to the aerosol induced by nebulizing 10 ml of suspension containing 10^{5-8} virulent H37Rv for 60 min. 4 wk after challenge infection all mice were killed and the number of virulent organisms present in their tissues determined. The results are illustrated in Fig. 2.

As seen in Fig. 2, all vaccinated animals exhibited increased resistance to challenge infection. The degree of resistance was unaffected by the age at which animals were vaccinated. Thus, tissues of mice in which extensive vaccinal multiplication had taken place because they were vaccinated at birth were no more resistant to the growth of challenge organisms than were tissues of mice vaccinated as adults in which BCG multiplication had not occurred.

TABLE IV
Multiplication of BCG in Organs of Mice Infected by Aerosol at Different Ages

Age when infected*	Time after injection	Number of bacilli present in organ† (log)	
		Lung	Spleen
3 wk	1 day	0	0
	11 days	2.6 ± 0.6	0
	3 wk	4.3 ± 0.2	0
	4 wk	5.8 ± 0.9	1.2 ± 1.0
	6 wk	6.1 ± 0.5	3.3 ± 1.1
	10 wk	5.3 ± 0.4	3.7 ± 1.1
	20 wk	3.1 ± 0.3	3.3 ± 0.6
	1 yr	1.3 ± 0.9	3.0 ± 1.1
1 day	1 day	0	0
	11 days	1.9 ± 1.2	0
	3 wk	3.8 ± 0.4	0.1 (2/5)
	4 wk	4.4 ± 1.0	1.1 ± 0.7
	6 wk	5.2 ± 0.4	2.1 ± 1.9
	10 wk	4.1 ± 0.3	4.0 ± 1.1
	20 wk	1.5 ± 0.8	2.7 ± 0.8
	1 yr	1.8 ± 1.0	3.0 ± 1.0

* Mice were infected by exposure to aerosol obtained by nebulizing 10.5 ml of suspension containing 10^6 viable units BCG/ml over a 60 min period.

† Figures refer to number of BCG colonies (expressed in logs) obtained from 1 ml (out of 5) of organ homogenate at given time after aerosol vaccination ± standard deviation. Also see Table I.

Protective Resistance of Mice Given Aerosol Vaccination.—Previous work has shown that BCG multiplies extensively in pulmonary and splenic tissue of adult mice when it is administered by the aerosol route. The following experiment deals with the intraorgan behavior of BCG after aerosol vaccination and the degree of resistance to virulent challenge infection induced thereby. Also included, for comparative purposes, is the BCG behavior and protective resistance induced in neonatal mice vaccinated by the aerosol route.

Newborn and 3-wk old female NCS mice were placed in separate baskets of the airborne infection apparatus and exposed for 1 hr to the aerosol induced by nebulizing 10.5 ml of a suspension containing 10^6 viable units of BCG bacilli/ml. At intervals after infection, mice were

killed and the numbers of vaccine bacilli contained in their spleens and lungs were ascertained. 10 wk after vaccination, animals of both groups, along with comparable untreated animals, were superinfected with virulent human bacilli given by the respiratory route. To this end animals were exposed for 60 min to the aerosol obtained by nebulizing 10 ml of suspension containing $10^{6.8}$ ml H37Rv/ml. They were sacrificed 4 wk after challenge and the number of virulent bacilli present in the animals' organs determined. The results of a typical experiment are given in Tables IV and V.

As seen in Table IV, BCG administered by aerosol grew extensively in the tissues of both newborn and adult animals. BCG colonies could not be obtained from organs of mice 1 day after infection. 10 days later, however, colonies were

TABLE V
Colonies of Virulent Mycobacteria Recovered from Organs of Mice Vaccinated by the Aerosol Route

Initial age	Vaccination	BCG present 10 wk after vaccination at the time of challenge* (log)		Colonies of virulent bacilli obtained from organs 4 wk after virulent aerosol infection*† (log)	
		Lung	Spleen	Lung	Spleen
3 wk	Control	—	—	6.8 ± 0.5	4.0 ± 0.3
	Vaccinated	5.3	3.7	5.5 ± 0.5§	2.2 ± 0.5§
1 day	Control	—	—	6.7 ± 0.4	3.7 ± 0.6
	Vaccinated	4.1	4.0	5.6 ± 0.8§	2.8 ± 0.2§

* Figures refer to number of BCG or H37Rv colonies (expressed in logs) recovered from 1 ml (out of 5) of organ homogenate at given time after vaccination or challenge infection ± standard deviation. See Table IV for details of vaccination. Also see Table I.

† Animals were challenged by exposure to virulent aerosol challenge (10 ml containing $10^{6.8}$ viable units H37Rv/ml for 60 min) 10 wk after vaccination.

§ Indicates values significantly different ($P = <0.05$) from those of comparable control mice.

obtained from the lungs of all animals. The number of colonies obtained increased until the 6th wk after infection, after which they began to decline. BCG organisms secondarily appeared in the spleens 3–4 wk after aerosol infection and increased in number through the 10th wk. The numbers stabilized or declined slightly thereafter. 1 yr after infection, significant numbers of bacilli could still be recovered from both splenic and pulmonary tissues.

The resistance of aerosol-vaccinated mice to virulent superinfection is illustrated in Table V. As seen, fewer virulent organisms developed in the tissues of vaccinated mice than in organs of unvaccinated mice. Importantly, the relative resistance attained by adult animals vaccinated by the aerosol route was entirely similar to that attained by mice vaccinated by the peritoneal route as described in Fig. 2. The lungs and spleens of aerosol-vaccinated adult mice, in which vaccine multiplication had occurred, contained respectively, 1.3 and 1.8 logs fewer challenge organisms than control mice. The corresponding figures

for peritoneally vaccinated mice in which BCG had not multiplied were 2.0 and 1.5 fewer bacilli.

The degree of resistance developed by the vaccinated mice was also the same whether the animals were exposed to BCG as babies or as adults (Table V). In the case of aerosol vaccination, as with peritoneal vaccination, the age at which an animal was vaccinated did not appear to affect its capacity to develop immunoprotection.

Growth of Normal Mice and Their Morphological Development.—The ability to restrain the infection induced by peritoneal administration of BCG developed abruptly between the 7th and 14th day of life. This corresponds to a time when many other changes occur in the physiological and morphological development of mice. It is during this time, for example, that mice begin to eat solid food and to develop their characteristic bacterial flora (4, 5). More importantly, it is also the time of maturation of the mouse's lymphoid tissues and the concomitant development of cellular immunological competence (6). The early growth rate and organ development of normal mice was determined in the following experiment.

Total body weights were determined at various ages from five normal animals and the animals were then killed by cervical dislocation. Lungs, spleens, and thymus were removed, stripped of advential tissue, and immediately weighed. In the case of nursing young, animals of both sexes were randomly selected from separate litters to minimize any genetic effects. 3-wk and older animals were females and were also randomly selected from different cages. The results of this experiment are given in Table VI.

The total body weights, as given in Table VI, yield a typical growth curve with an approximately linear increase in size through weaning (21 days), and a progressively decreasing rate of weight change thereafter.

The lungs of these mice had attained 20% of their mature animal weight when the animal was 1 day old and 45% of their mature weight by the 7th day of life. Pulmonary tissue had also attained its normal adult relative size, in milligrams of lung per gram of animal body weight, by the first day of life. In contrast, splenic tissue had attained only 1–2% of its adult weight by the 1st day after birth and only 27% by the 7th day of life. Its adult relative weight was not attained until the 7th day of life. Similarly, thymic tissue increased in size slowly until the 10th day after birth. By this time, however, it had attained its heaviest relative weight. Maximum size was reached at the time of weaning. Thereafter, both actual and relative size of the thymus decreased.

The relative increase in the numbers of BCG which occurred in spleens and lungs during this time is of interest. Splenic tissue increased approximately 50 times in size during the first 6 wk of an animal's life. During this period of time, the numbers of BCG in spleens of animals vaccinated when they were 1 day old increased more than 1000 times. Similarly, while pulmonary tissue increased fivefold over the same period, the corresponding bacillary increase in lungs of

neonatically vaccinated mice was of the order of 2500 times. In both tissues there was therefore a true net bacillary multiplication rather than merely an increase in numbers of bacilli to parallel the increasing size of tissue.

Effect of BCG on Growth of Animals and Tissue Development.—As already mentioned, there is extensive multiplication of BCG administered to newborn mice. It has been reported that this strain of BCG also induces marked changes in the food intake and nitrogen excretion of adult mice (7). It was of interest therefore to determine whether the growth rate of neonatal animals was altered by BCG infection.

TABLE VI
Weight of Normal Mice and Their Organs at Different Ages

Age	Total body weight	Lung			Spleen			Thymus		
		Total weight	Relative* weight	% Adult† weight	Total weight	Relative weight	% Adult weight	Total weight	Relative weight	% Adult weight
days	g	mg	mg/g		mg	mg/g		mg	mg/g	
1	1.8 ± 0.1§	29 ± 5	16	19	1 ± 0.1	0.6	2	3 ± 0.3	1.7	4
7	3.4 ± 0.5	68 ± 5	20	45	16 ± 8	4.7	27	5 ± 4	1.5	7
10	7.9 ± 0.2	128 ± 5	16	85	46 ± 3	5.8	77	51 ± 7	6.5	75
14	8.2 ± 0.6	119 ± 5	15	80	38 ± 8	4.6	64	49 ± 7	6.0	72
21	11.8 ± 0.3	131 ± 16	11	87	97 ± 12	8.2	160	86 ± 11	7.3	126
40	17.7 ± 0.2	150 ± 21	8	(100)	60 ± 16	3.4	(100)	68 ± 7	3.8	(100)
180	32.0 ± 1.2	207 ± 33	6	139	128 ± 40	4.0	214	56 ± 8	1.8	82

* Relative weight = mg tissue per gram total body weight.

† Adult organ weight arbitrarily taken as that value obtained for 40 day old animal; i.e., at the time of sexual maturity.

§ All figures correspond to the average for 5 animals in each group ± standard deviation. Wet weights of organs obtained immediately after separation from the animal.

Several litters of newborn animals were pooled. One group of animals was vaccinated by exposure to an aerosol obtained by nebulizing 10 ml of suspension containing $10^{5.5}$ viable BCG per ml over a 60 min period. A second group was peritoneally injected with 0.05 ml of vaccine containing $10^{6.2}$ organisms. A third group received 0.05 ml of physiological saline by intraperitoneal injection while a fourth group was left untreated. Animals were segregated by sex and returned to randomly selected foster mothers after treatment. Eight animals, four male and four female, were placed with each nursing female. Animals were weaned at 21 days. Total body weights were obtained on individual animals at several intervals after their birth. The results are given in Table VII.

No mortality occurred in any group of animals for at least a year after treatment. As seen in Table VII, animals injected by the peritoneal route, whether by saline or BCG, had slightly depressed weights at several periods of time. These depressions were never large, however, and indeed are of doubtful significance. Other animals grew normally.

The effect of peritoneal injection of BCG in newborn mice on the subsequent growth of lung, spleen, and thymus was also determined in similar experiments. No significant effect due to vaccine infection was noted. The total and relative sizes of these organs were the same in BCG-treated and in control animals at all intervals after infection. Splenomegaly did not occur.

DISCUSSION

Animals vaccinated with BCG are significantly protected against virulent challenge infection (1, 8-10). Living organisms generally are found to induce a

TABLE VII
Effect of BCG Infection on Growth of Mice

Treatment*	Sex†	Weight of animals at given age (days)					
		1	10	21	42	84	231
Control	Male	1.7‡	6.9	13.6	31.1	39.2	45.5
Control (saline)		1.7	6.3	12.0	27.5	35.9	43.4
BCG (aerosol)		1.7	6.5	12.6	28.8	38.8	49.3
BCG (peritoneal)		1.7	5.8	12.5	28.2	35.8	43.2
Control	Female	1.7	6.7	12.9	25.7	33.7	41.2
Control (saline)		1.7	6.7	12.2	24.7	31.8	43.4
BCG (aerosol)		1.7	6.5	12.6	25.5	35.1	42.5
BCG (peritoneal)		1.7	5.8	12.0	24.8	30.4	38.9

* All treatments given when animals were 1 day old. Physiological saline was given peritoneally in volume of 0.05 cc. BCG given aerosol; 10 ml suspension containing 10^6 viable units per ml over a 60 min period. BCG given peritoneally; $10^{6.2}$ viable units per mouse in volume of 0.05 ml.

† Animals sexed at birth and four males and four females nursed per foster mother.

‡ Figures are average for six to eight litters of mice through the 21st day, or for 14-28 adult animals.

greater degree of antitubercular immunity than do killed bacilli. There is controversy, however, regarding the relative effectiveness of immunity induced by living nonmultiplying vaccines as contrasted with living and multiplying organisms (11).

Several studies have indicated that in vivo multiplication is not necessary for obtaining an immune response in mice (7, 12-16). Previous studies from this laboratory, for example, have revealed that induction of immunity depends on vaccinal multiplication only when the amount of BCG administered is very small. With large vaccine doses the amount of bacterial protoplasm injected provides outright an antigenic mass sufficient to elicit a high level of antitubercular resistance (14).

The experimental results reported in this paper extend these findings. Thus

it was found that BCG administered by the peritoneal route multiplied extensively in newborn mice, whereas little bacillary growth occurred when the same vaccine was given to adult mice. Yet both groups of vaccinated animals became equally protected against virulent respiratory superinfection. Moreover, extensive multiplication of BCG occurred in either neonatal or adult mice when the vaccine was administered by the aerosol route. Animals vaccinated by this route, however, became no more resistant to virulent challenge infection than did adult mice vaccinated by the peritoneal route.

It should be noted that these conclusions may apply only to immunity against the growth of virulent bacilli administered by the respiratory route. As noted elsewhere (2) protection against this form of challenge differs in several important respects from that against the lethal aspects of intravenous tubercular infection. It is possible, therefore, that the mice vaccinated at different ages may respond differently if tested by other challenge methods.

The mechanisms through which adult animals restrain the *in vivo* multiplication of peritoneally injected mycobacteria is not known. As reported in this paper, the extent of restraint depends on the strain of mycobacteria used. Relatively avirulent BCG are inhibited by the animals' tissues whereas virulent human mycobacteria are not. On the other hand, it has been shown elsewhere that different strains of peritoneally injected BCG do not significantly differ in their ability to elicit tissue tuberculostasis (1).² Moreover, neither the metabolic condition of the vaccine, *i.e.* whether in a state of active growth or not, nor the size of the inoculum significantly influences the *in vivo* growth of peritoneally administered bacilli.

It has also been found that neither the age of adult animals nor their dietary history significantly alters the behavior of peritoneally injected BCG. In contrast, as revealed in the studies reported here, the tissues of immature animals have little ability to restrain the growth of peritoneally administered vaccinal organisms.

The capacity to restrain vaccine organisms develops abruptly in mice between the 7th and 14th day of postnatal life. The lymphoid system of mice is known to be highly immature when the animal is born (17). While the thymus is clearly differentiated at birth, little development of peripheral lymphoid tissue has occurred; the spleen has not differentiated into typical pulp areas and lymphocytes have not aggregated into its follicles. Importantly, while the spleen of newborn animals is capable of harboring intact bacteria it has not yet developed antigen-retaining structures or immunological competence. These functions develop (in rats) only 12–14 days after birth (18, 19).

² However, strains of BCG do differ in their ability to multiply *in vivo* after intravenous or aerosol infection. These differences are correlated with the ability of different BCG vaccines to induce antitubercular protection in animals (12) and according to unpublished reports also in man.

Not all organ systems of mice are equally immature at the time of birth (20). Pulmonary tissue, for example, is fully functional at this time although some morphological and metabolic changes occur during postnatal growth (21, 22). Nonetheless, the pulmonary tissue of newborn animals is no more able than splenic tissue to suppress the growth of peritoneally injected BCG. The tuberculostatic capabilities appear to mature first in splenic tissue and later in the animal's lung. This suggests that vaccinal dormancy is related to the development of lymphoid tissue and may, as such, reflect a state of rapidly acquired antitubercular resistance.

SUMMARY

The consequences of Calmette-Guérin bacillus (BCG) vaccination were followed in newborn and adult mice.

BCG failed to multiply in the organs of adult mice when administered peritoneally. In contrast, extensive multiplication of the vaccine occurred in both splenic and pulmonary tissue after its peritoneal administration to newborn mice. This absence of tuberculostasis occurred during the period when the animal's spleen, lung, and thymus were rapidly growing.

Animals achieved similar levels of resistance to virulent respiratory challenge 10 wk after vaccination irrespective of whether the vaccine had been administered when the mice were newly born and BCG had multiplied in vivo or administered when the animals were fully grown and the BCG had remained in the dormant state.

Although neonatal infection with BCG was severe, as shown by the large numbers of organisms recovered from the animals' tissues, the animals suffered no mortality or overt signs of disease. Neonatal vaccination did not significantly affect either the animal's growth rate or the gross development of its organs.

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