AN EXPERIMENTAL STUDY OF PROTECTIVE INOCULATION WITH HEAT KILLED TUBERCLE BACILLI*

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The purpose of these experiments has been to determine if inoculation with tubercle bacilli killed by heat is an effective means of protection against tuberculous infection and to compare the relative value of killed and attenuated (BCG) tubercle bacilli. Furthermore, killed tubercle bacilli have been injected in combination with a variety of agents in the attempt to increase their immunizing activity.

Calmette (1) has maintained the opinion that killed tubercle bacilli have only feeble protective power. No increased resistance was observed by Theobald Smith (2) when he attempted to immunize cattle with tubercle bacilli killed by heat and none was found in guinea pigs by Uhlenhuth (3), by Dold (4) and by Dienes and Schoenheit (5). Guinea pigs inoculated by Petroff and Stewart (6), and by Zinsser, Ward and Jennings (7), with large quantities of heat killed tubercle bacilli lived somewhat longer after infection than control animals with no preparatory inoculation. The parenteral treatment of guinea pigs by B. Lange, R. Freund and Jochimsen (8) with heat killed (100°C.) tubercle bacilli increased slightly their resistance to intracutaneous infection.

Guinea pigs were given by Pagel (9) eight to ten intraperitoneal injections of 1 mg. human tubercle bacilli killed by heat, and subsequently infected by intracutaneous injection of virulent human tubercle bacilli. A considerable part of the animals, killed after 3 months, were in some degree protected against the disease, but when a large infecting dose was used protection was, he believed, greater with broth alone than with heat killed tubercle bacilli combined with broth.

When tubercle bacilli were administered to rabbits intracutaneously or subcutaneously B. Lange, Jochimsen and Magat (10) found that the severity of tuberculous infection produced by intratracheal inoculation was diminished though the disease was never prevented. Rabbits were subjected by Westenrijk

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(11) to the inhalation of a vaporized suspension of heat killed tubercle bacilli. When animals that had received this treatment were infected by subcutaneous injection of virulent tubercle bacilli and killed 3 months later, tuberculosis was in some instances just as widespread as in control untreated animals, but in other instances there was evidence of protection. Rabbits vaccinated by Thomas (12) with tubercle bacilli killed by exposure to formalin acquired no protection, but those treated with tubercle bacilli killed by heat at 70°C. for 1 hour survived infection approximately half as long again as their controls.

Dissemination of bovine tubercle bacilli from the site of injection was retarded in guinea pigs treated by J. Freund (13) with heat killed tubercle bacilli; the same retardation of dissemination was observed by Saenz (14) in guinea pigs that had been injected with killed tubercle bacilli suspended in oil of vaseline.

The production of antibodies in response to the injection of heat killed tubercle bacilli was demonstrated by Hughes (15) in rabbits "vaccinated" by repeated injections of tubercle bacilli subjected to a temperature of 100°C. for 1 hour. He found that progressively increasing bacteriotropic action of the serum promoted phagocytosis of both mononuclear cells and polymorphonuclear leukocytes.

Infants were injected intravenously by Langer (16) with heat killed tubercle bacilli; of ten infants that received three simultaneous injections into one forearm and 4 weeks later three injections into the other forearm, nine became sensitive to tuberculin. Similar observations have been made by Klotz and Sänger (17) and by Fedders (18). During 7 years Raw (19) administered heat killed human tubercle bacilli to more than 400 children with tuberculous parents, but in the absence of controls was unable to determine if resistance to the disease was increased. Of seventeen infants and nine young children "vaccinated" by Zadek and Meyer (20) all became sensitive to tuberculin within about 2½ months. Goodwin and Schwentker (21) gave 73 newborn infants or young children from one to four intramuscular injections of human tubercle bacilli killed by heat at 100°C. in doses of 0.03 to 0.1 mg.; all but one became sensitive to tuberculin. In nine children sterile cold abscesses formed at the site of injection. It was not possible to maintain adequate controls and no definite increase of resistance was demonstrable.

Methods

In the experiments that will be described rabbits have been used because they are much more resistant to infection with human or bovine tubercle bacilli than guinea pigs. Fortunately it was learned early in the progress of these experiments that skin sensitization of rabbits can be measured with approximate accuracy by the use of tuberculin or of killed tubercle bacilli injected intracutaneously. In later experiments complement fixation was used to determine the progress of antibody formation.

The killed tubercle bacilli employed in these experiments were prepared from either bovine or human tubercle bacilli, in great part from the former. The strain of bovine tubercle bacillus (Ravenel) that was used was isolated from a cow by Dr. Mazyck Ravenel more than 20 years ago; it was highly virulent for rabbits and guinea pigs and was used to infect rabbits. A strain of human tubercle bacillus (A_1D) isolated at The Henry Phipps Institute a few years ago was used, both living and killed, to immunize rabbits.

The bacillus of Calmette and Guérin (BCG) was originally obtained from the Pasteur Institute in Paris and propagated on glycerin-bile-potato and on the synthetic medium of Sauton according to the procedure recommended by Calmette (22) for human vaccination.

Tubercle bacilli were grown on glycerin agar for 4 to 6 weeks but the usual medium containing proteins of egg was not used in order to exclude the possibility of sensitization with egg protein. Bacteria removed from the agar slants were weighed, ground in a mortar and suspended in salt solution so that 1 cc. of the suspension contained 10 mg. of tubercle bacilli. The suspension was kept at 100°C. in an Arnold steam sterilizer for half an hour. After the suspension had cooled to room temperature, 0.35 per cent tricresol was added. Each lot of "vaccine" was tested for the presence of viable tubercle bacilli by injecting 0.5 cc. of it into the subcutaneous tissue of the left groin of each of six guinea pigs. The guinea pigs were killed from 4 to 6 weeks after the injection and examined for tuberculosis. In some later experiments "vaccine" heated at 60°C. for half an hour was used. A suspension of living tubercle bacilli was put into a flask and its neck was sealed by melting; the flask was submerged for half an hour in water at 60°C.

The intravenous injection of bovine tubercle bacilli produces in rabbits lesions that are predominantly pulmonary. The disease is progressive and fatal, the duration of life after infection varying with the quantity of the infecting microorganisms. It is evident that the relation of virulence of the infecting bovine tubercle bacillus to the resistance of the rabbit does not reproduce the accurate adjustment that exists between the human tubercle bacillus and the human host. Though tuberculosis of man often produces progressive fatal disease this adjustment favors the host so that most of those who are infected recover. Neither human nor bovine tubercle bacilli are similarly adjusted in virulence to any available animal.

Survival of animals treated with a protective agent is doubtless the best measure of their resistance. Since the rabbit is more susceptible to the bovine bacillus than man to the human, protection with recovery is much less likely to occur in the rabbit than in man, when the

two receive comparable methods of protective inoculation. The use of animals more resistant to tuberculosis, namely, mice, rats, cats, dogs or goats, for testing protection, is inadvisable because they are naturally far more resistant than man. It is evidently desirable to find an animal with resistance to tubercle bacilli comparable to that of man to the tubercle bacillus of human type. In the experiments that will be described some approximation to this relation has been obtained by the use of the brown Havana rabbit, which is more resistant to the bovine tubercle bacillus than the other breeds in common use. The infecting dose of tubercle bacilli, namely, 0.00001 mg., used in the experiments has been uniformly fatal to animals of this breed, but the proportion of those that survive after protective inoculation is considerably greater than with the other breeds.

The difficulties of experiments undertaken to measure resistance by survival from infection are considerable because it is necessary to maintain a large number of animals under uniform conditions during a period of approximately 2 years. The most trustworthy results are doubtless obtainable when a considerable number of animals, subjected to the procedures under investigation, are inoculated with the same suspension of tubercle bacilli and compared with a group of controls similarly infected; hence the number of animals in some of the experiments was 150, and two or more experiments were usually in progress at the same time. The possibility of spontaneous reinfection of rabbits (Lurie (23)) makes it essential to keep each animal in a separate cage. It is probable that a larger proportion of animals would survive infection if it were possible to maintain them in quarters that permitted natural exercise. Another factor that is controlled with much difficulty is the occurrence of infection with *Bacillus lepisepticus* producing "snuffles" and often fatal bronchopneumonia. This danger unfortunately increases with the duration of life so that protected animals have more opportunity to acquire infection than controls.

After experience had shown that a considerable number of animals that survived infection during 2 years had no demonstrable anatomical lesions, the experiments were ended after this interval and all animals were then killed and examined.

In experiments undertaken to protect guinea pigs against tuberculosis, the duration of life after inoculation must be used as the chief

criterion of protection because these animals seldom, if ever, acquire sufficient resistance to ensure their recovery. In the experiments of Petroff and Stewart (6), inoculation of killed tubercle bacilli prolonged the average life after infection from 147 to 193 days but none survived infection. Though recovery from the disease is the best criterion of resistance conferred by protective inoculation, the duration of life of animals that succumb may have considerable value as evidence of resistance.

To avoid the difficulty of maintaining a large number of animals during a long period of observation, some experimental studies have been terminated a few months after infection and the extent of tuberculous lesions has been used as a measure of resistance. This procedure may give doubtful results because numerous observations including our own have shown that lesions may be more extensive, for a time at least, in previously sensitized than in control animals, whereas at a later period the lesions diminish with healing. Later resolution with partial or complete healing may occur.

Experiment A.—Immunization was subcutaneous with 5 injections at intervals of 5 days. The injections were as follows:

Group 1. 0.02 mg. of living human tubercle bacilli suspended in 0.1 cc. salt solution.

Group 2. 0.02 mg. heat killed bovine tubercle bacilli suspended in 0.1 cc. salt solution.

Infection (see group 3) was intravenous with 0.01 mg. bovine tubercle bacilli given 9 days after the last injection of vaccine.

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life after infection of animals with tuberculosis
				days
1	Living human tubercle bacilli	5	0	87
2	Heat killed bovine tubercle bacilli	5	0	58.4
3	Infected controls	5	0	70.6

Experiment B.—Immunization was subcutaneous with repeated injections at intervals of 4 or 5 days, as follows:

Group 1. Rabbits received in 6 injections bovine tubercle bacilli (Bov. C), killed by heat at 100°C.

Group 2. Rabbits received 4 subcutaneous injections of 0.5 cc. horse serum; 5 days after the last injection, 0.2 cc. horse serum produced acute inflammation with necrosis of the skin (Arthus phenomenon). Later at intervals of 5 days the animals received 6 injections each containing 0.02 cc. heat killed tubercle bacilli and 0.1 cc. horse serum.

Infection (see group 3) was intravenous with 0.001 mg. bovine tubercle bacilli given 5 days after the last injection of vaccine.

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life of animals with tuberculosis
				days
1	Heat killed tubercle bacilli	5	0	98
2	Heat killed tubercle bacilli and horse serum	9	0	80.8
3	Infected controls	6	0	47.5

Experiment C.—Immunization was subcutaneous with 11 injections at intervals of 4 or 5 days. The injections were as follows:

Group 1. 10 injections with 1 mg. bovine tubercle bacilli killed by heat at 100°C. and 1 injection with 0.2 mg. heat killed tubercle bacilli given as a skin test.

Group 2. 10 injections with 0.2 mg. of heat killed bovine tubercle bacilli and 1 skin test as in the preceding group.

Group 3. 10 injections with 0.1 mg. of heat killed bovine tubercle bacilli and 1 skin test.

Group 4. 5 injections with 0.5 cc. undiluted horse serum, 1 injection with 0.2 mg. horse serum as a skin test (now sensitive to horse serum) and 5 injections of 0.2 mg. of heat killed tubercle bacilli mixed with 0.2 cc. horse serum.

Group 5. 5 injections with 0.5 cc. of a mixture of equal parts of horse serum and 0.5 per cent sodium carbonate solution heated for a half hour to 100°C., 1

Experiment C

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life of animals with tuberculosis
				days
1	Heat killed tubercle bacilli (1 mg.)	10	0	135
2	Heat killed tubercle bacilli (0.2 mg.)	5	0	111.8
3	Heat killed tubercle bacilli (0.1 mg.)	5	0	110
4	Horse serum and heat killed tubercle bacilli	5	0	118
5	Heated horse serum and heat killed tubercle bacilli	6	0	139
6	Typhoid vaccine and heat killed tubercle bacilli	6	0	109
7	Infected controls	19	0	95

injection of 0.2 cc. of diluted and heated horse serum (animals now sensitive to heated horse serum) and 5 injections of 0.2 mg. heat killed tubercle bacilli mixed with 0.4 cc. diluted and heated horse serum.

Group 6. 6 subcutaneous injections with 0.5 or 1 cc. commercial typhoid vaccine (heat killed typhoid bacilli) followed by 5 subcutaneous injections with 0.2 mg. heat killed tubercle bacilli mixed with 0.5 cc. typhoid vaccine.

Infection was intravenous (see group 7) with 0.001 mg. bovine tubercle bacilli given 1 week after the last immunizing injection.

Experiment D.—Immunization was subcutaneous with 11 injections at intervals of approximately 1 week. The injections were as follows:

Group 1. 1 mg. bovine tubercle bacilli killed by heat at 100°C.

Group 2. 1 mg. human tubercle bacilli killed by heat.

Group 3. 1 mg. BCG.

Group 4. 1 mg. heat killed bovine tubercle bacilli and 0.9 cc. of a mixture of equal parts of horse serum and of 0.5 per cent sodium carbonate heated at 100°C. for a half hour.

Group 5. 1 mg. heat killed bovine tubercle bacilli and 0.9 cc. commercial typhoid vaccine.

Infection (see group 6) was intravenous with 0.0001 mg. bovine tubercle bacilli given 8 days after the last vaccinating injection.

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life after infection of animals with tuberculosis
				days
1	Heat killed bovine tubercle bacilli	25	0	133
2	Heat killed human tubercle bacilli	24	0	130.7
3	BCG	24	0	167.3
4	Heat killed bovine tubercle bacilli and heated horse serum	25	1	168.2
5	Heat killed bovine tubercle bacilli and typhoid vaccine	25	0	172
6	Infected controls	25	0	131.9

Experiment E (Figs. 1 and 2).—Immunization was subcutaneous with 15 injections at intervals of 2 or 3 days. These injections were as follows:

Group 1. 0.4 mg. of heat killed bovine tubercle bacilli suspended in 0.4 cc. salt solution.

Group 2. 0.4 mg. heat killed human tubercle bacilli.

Group 3. 0.4 mg. of BCG.

Group 4. 0.4 mg. of heat killed bovine tubercle bacilli and 0.36 cc. of a mixture of equal parts of horse serum and 0.5 per cent solution of sodium carbonate heated for a half hour at 100°C.

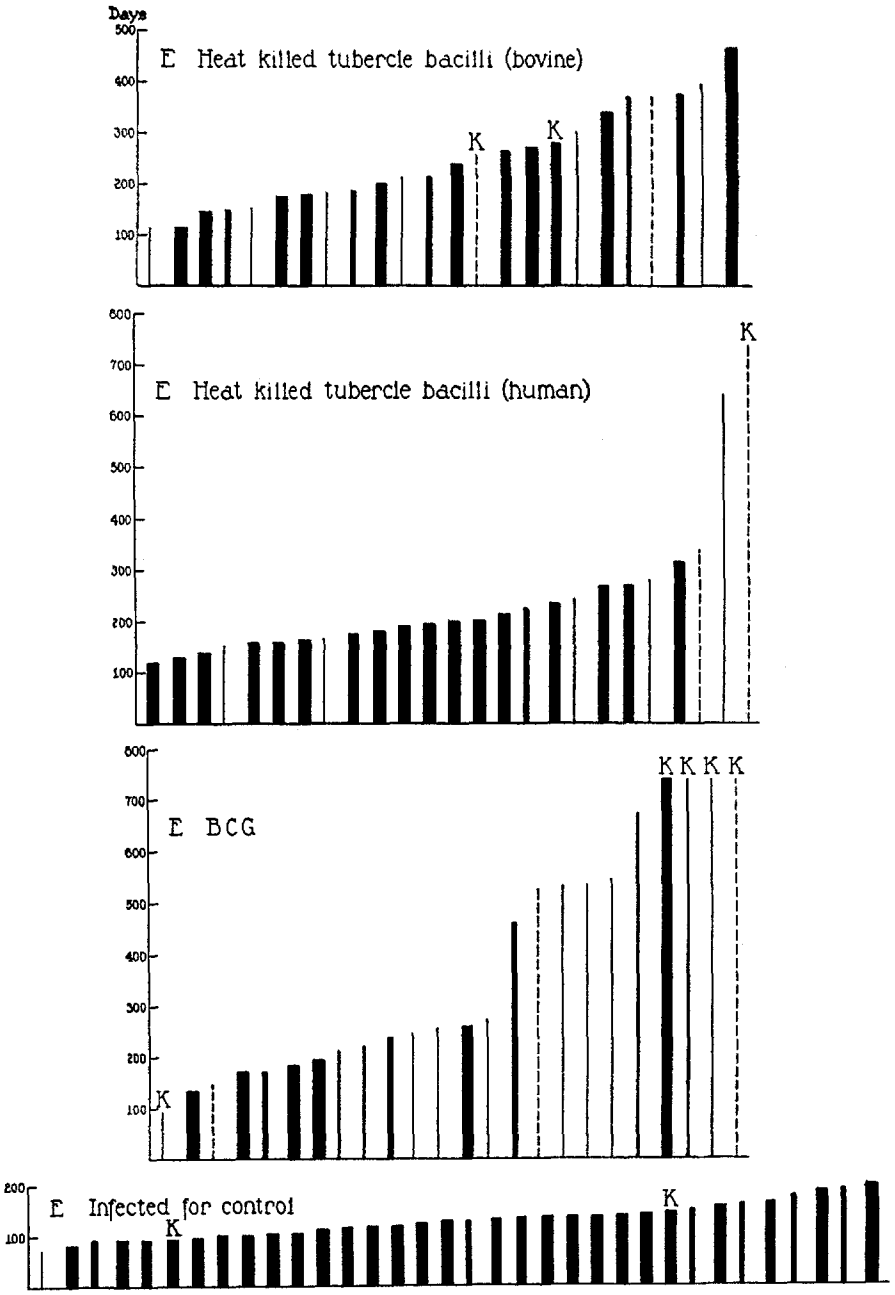

FIG. 1. In this and in subsequent figures each rabbit is represented by a vertical line. The length of this line shows the duration of life in days after infection. The width of the line indicates the relative extent of tuberculosis of the lung, the broadest line representing approximately nine-tenths of the cut surface and the narrowest lines one-twentieth or less. In several animals represented by the narrowest line there was no tuberculosis of the lung and scant tuberculosis elsewhere. Broken lines indicate no tuberculosis. Immunized may be compared with control animals. See description of Experiment E.

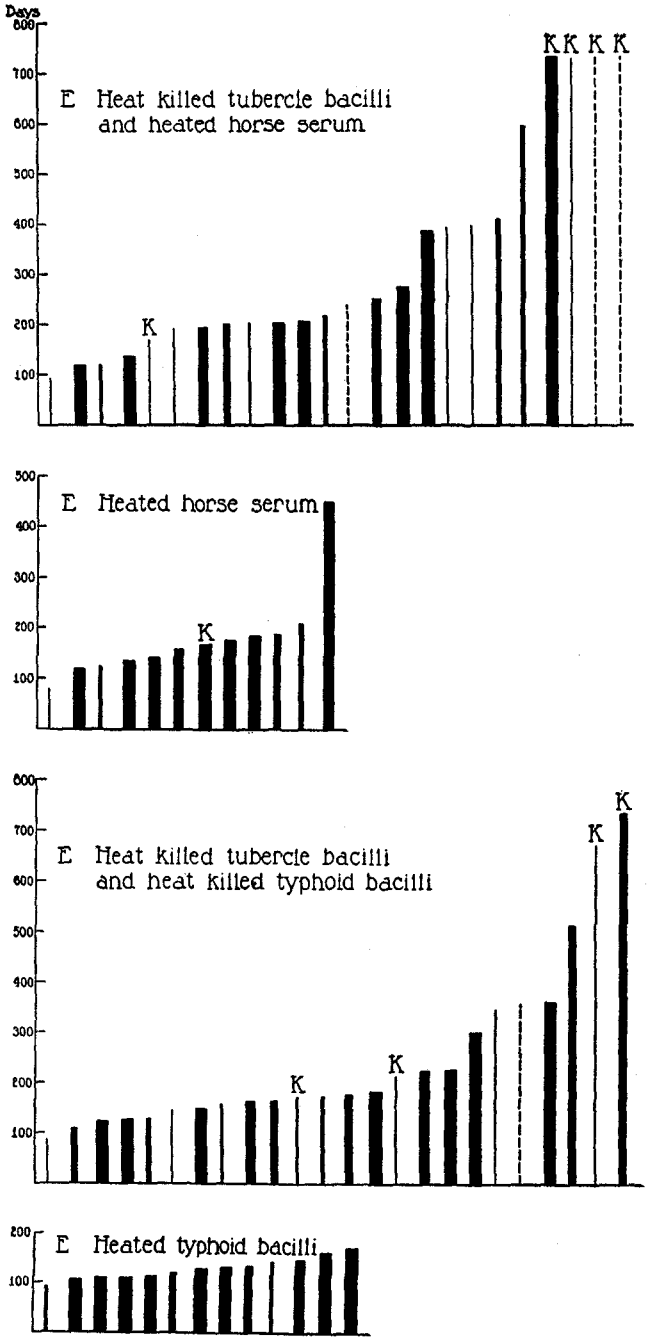

FIG. 2. See description of Experiment E.

Group 5. Heated horse serum alone in quantity equal to that used in group 1.

Group 6. 0.4 mg. of heat killed bovine tubercle bacilli mixed with 0.36 cc. of typhoid vaccine (heat killed typhoid bacilli).

Group 7. Typhoid vaccine alone in quantity equal to that given to the foregoing group.

Infection (see group 8) was intravenous with 0.00001 mg. bovine tubercle bacilli given 7 days after the last immunizing injection.

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life after infection of animals with tuberculosis
				days
1	Heat killed bovine tubercle bacilli	24	2	240.3
2	Heat killed human tubercle bacilli	25	2	217.8
3	BCG	24	3	369.5
4	Heat killed bovine tubercle bacilli and heated horse serum	24	3	296.6
5	Heated horse serum alone	12	0	183.1
6	Heat killed bovine tubercle bacilli with typhoid vaccine	24	1	245.5
7	Typhoid vaccine alone	13	0	127.7
8	Infected controls	34	0	129.3

Experiment F.—Immunization was intracutaneous with 6 injections at intervals of 1 week. Each injection was as follows:

Group 1. 0.4 mg. heat killed bovine tubercle bacilli.

Group 2. 0.4 mg. heat killed bovine tubercle bacilli and 0.1 cc. of a mixture of equal parts of sheep serum and 5 per cent sodium carbonate heated for a half hour at 100°C.

Group 3. 0.4 mg. heat killed bovine tubercle bacilli mixed with 0.6 cc. of a suspension of typhoid bacilli killed by heat at 60°C. for 45 minutes.

Infection (see group 4) was with 0.00001 mg. of bovine tubercle bacilli given 7 days after the last vaccinating injection.

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life after infection of animals with tuberculosis
				days
1	Heat killed bovine tubercle bacilli	14	1	293.5
2	Heat killed bovine tubercle bacilli and heated sheep serum	15	1	245.5
3	Heat killed bovine tubercle bacilli and heat killed typhoid vaccine	12	0	266.9
4	Infected controls	14	0	135.9

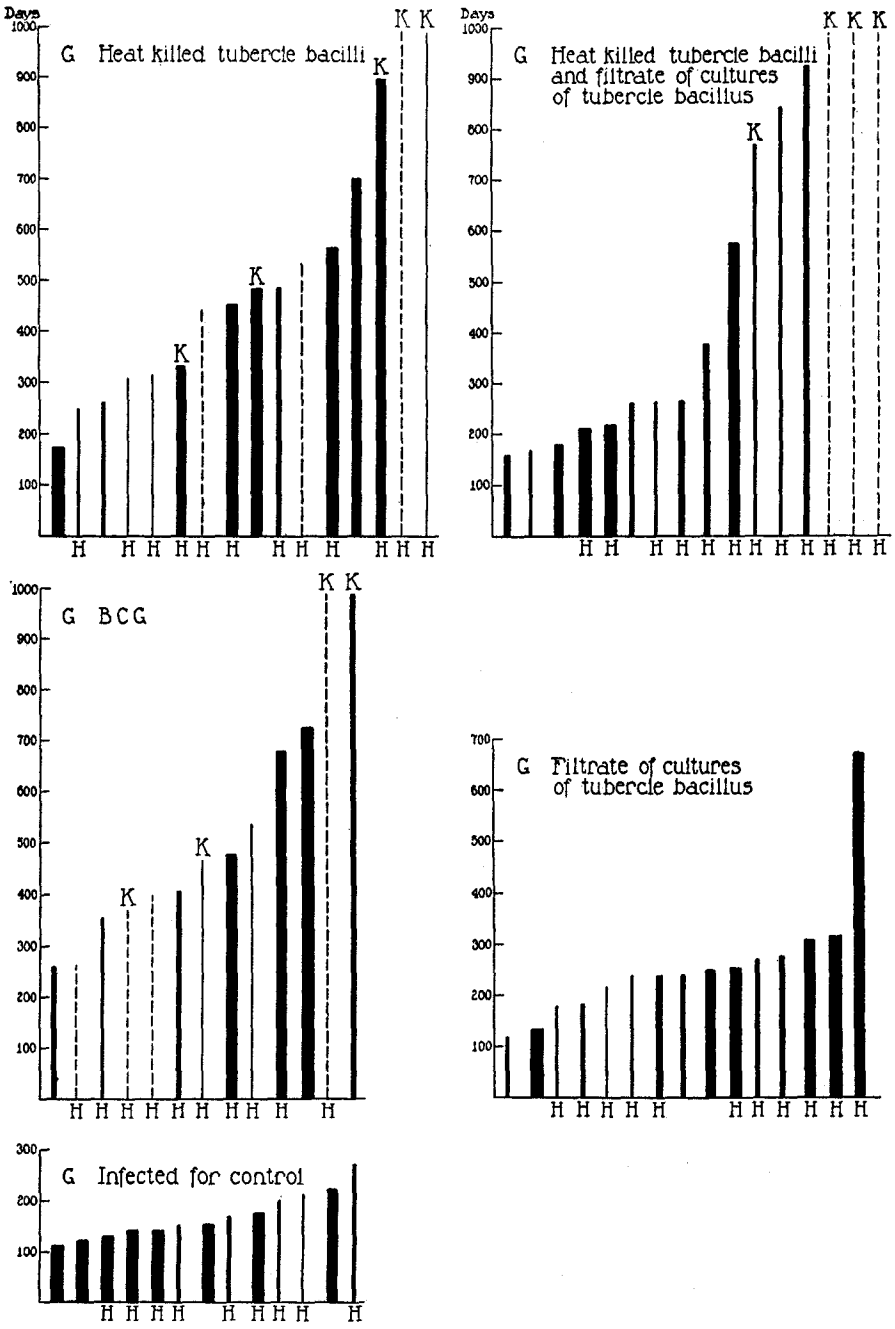

FIG. 3. See description of Experiment G. H indicates Havana rabbits.

Experiment G (Fig. 3).—In this experiment rabbits were partly Havana and partly other breeds. During the course of the experiment the diet of the animals was changed from oats and greens to a composite diet containing an excess of sugar; diarrhea followed and a considerable number of animals died. These were in part replaced as indicated below.

Immunization was intracutaneous with 8 injections given at intervals of 1 week; these injections varied with different groups.

Group 1. The first injection was with 0.5 mg. heat killed tubercle bacilli in 0.25 cc. salt solution; subsequent injections were with 0.25 mg. heat killed tubercle bacilli in the same volume of salt solution. 3 animals introduced into the experiment to replace animals that died during the course of vaccination received only 4 injections of 0.25 mg. heat killed bacilli; the average duration of life of these animals after infection was 481.7 days.

Group 2. The first injection was with 0.5 mg. BCG and subsequent injections with 0.25 mg. in 0.25 cc. salt solution. 2 animals introduced into the experiment after immunization had begun received only 4 injections of 0.25 mg. BCG; their average duration of life was 626.5 days.

Group 3. The first injection was with 0.5 mg. heat killed bovine tubercle bacilli mixed with 0.2 cc. of a filtrate from a culture of bovine tubercle bacilli, the volume being brought to 0.25 cc. with salt solution. In subsequent injections the quantity of heat killed tubercle bacilli was reduced to 0.25 mg. One animal that received only 4 injections, containing 0.25 mg. heat killed bacilli, lived 261 days.

Group 4. Filtrate from a culture of bovine tubercle bacilli was used alone.

Infection (see group 5) was with 0.00001 mg. of bovine tubercle bacilli given 11 days after the last vaccinating injection.

Group	Immunization with	Number of Havana rabbits	Number of Havana rabbits with no tuberculosis	Average length of life after infection of Havana rabbits with tuberculosis	Number of rabbits of other breeds	Number of rabbits of other breeds with no tuberculosis	Average length of life after infection of rabbits of other breeds dying with tuberculosis
				days			*days*
1	Heat killed bovine tubercle bacilli	12	3	510	4	0	404.7
2	BCG	10	4	488.7	3	0	592
3	Heat killed bovine tubercle bacilli and filtrate	12	3	494.4	4	0	191
4	Filtrate alone	11	0	285.8	4	0	183.2
5	Infected controls	9	0	178.2	4	0	153

Experiment I.—Immunization was with 20 injections given at intervals of approximately 1 week. These injections were as follows:

Group 1. Intracutaneous with 0.2 mg. bovine tubercle bacilli killed by heat at 100°C.

Group 2. Intravenous with 0.2 mg. of heat killed bovine tubercle bacilli.

Group 3. Intracutaneous with 0.2 mg. BCG.

Infection (see group 4) was with 0.00001 mg. bovine tubercle bacilli given 8 days after the last vaccinating injection.

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life after infection of animals with tuberculosis
				days
1	Heat killed bovine tubercle bacilli intracutaneously	9	0	188
2	Heat killed bovine tubercle bacilli intravenously	5	0	178.6
3	BCG			
4	Infected controls	9	0	124.5

Experiment J.—Immunization was intracutaneous with 9 injections at intervals of 1 week. The injections were as follows:

Group 1. 0.2 mg. of bovine tubercle bacilli killed by heat (100°C.) in 0.2 cc. salt solution.

Group 2. 0.2 mg. heat killed tubercle bacilli mixed with 1 mg. of turtle bacillus in 0.2 cc. salt solution.

Group 3. 0.2 mg. heat killed tubercle bacilli in 0.2 cc. 0.1 per cent solution of alum.

Infection was intravenous with 0.00001 mg. bovine tubercle bacilli given 1 week after the last vaccinating injection.

Group	Immunization with	Number of rabbits	Number with no tuberculosis	Average length of life after infection of animals with tuberculosis
				days
1	Heat killed bovine tubercle bacilli	5	2	245.6
2	Heat killed bovine tubercle bacilli and turtle tubercle bacilli	5	0	194.2
3	Heat killed bovine tubercle bacilli and alum	3	0	219.3

Experiment K (Fig. 4).—Immunization was intracutaneous with 12 injections at intervals of approximately 1 week.

Group 1. The first injection was with 0.5 mg. of bovine tubercle bacilli killed by heat at 60°C. in 0.3 cc. of salt solution; the second injection was with 0.25 mg., the third with 0.1, the fourth and subsequent injections with 0.025 mg. heat killed tubercle bacilli.

Group 2. The first injection was with 0.5 mg. BCG and in subsequent injections the quantities were the same as with group 1.

Group 3. Bovine tubercle bacilli killed by heat were injected in the same doses

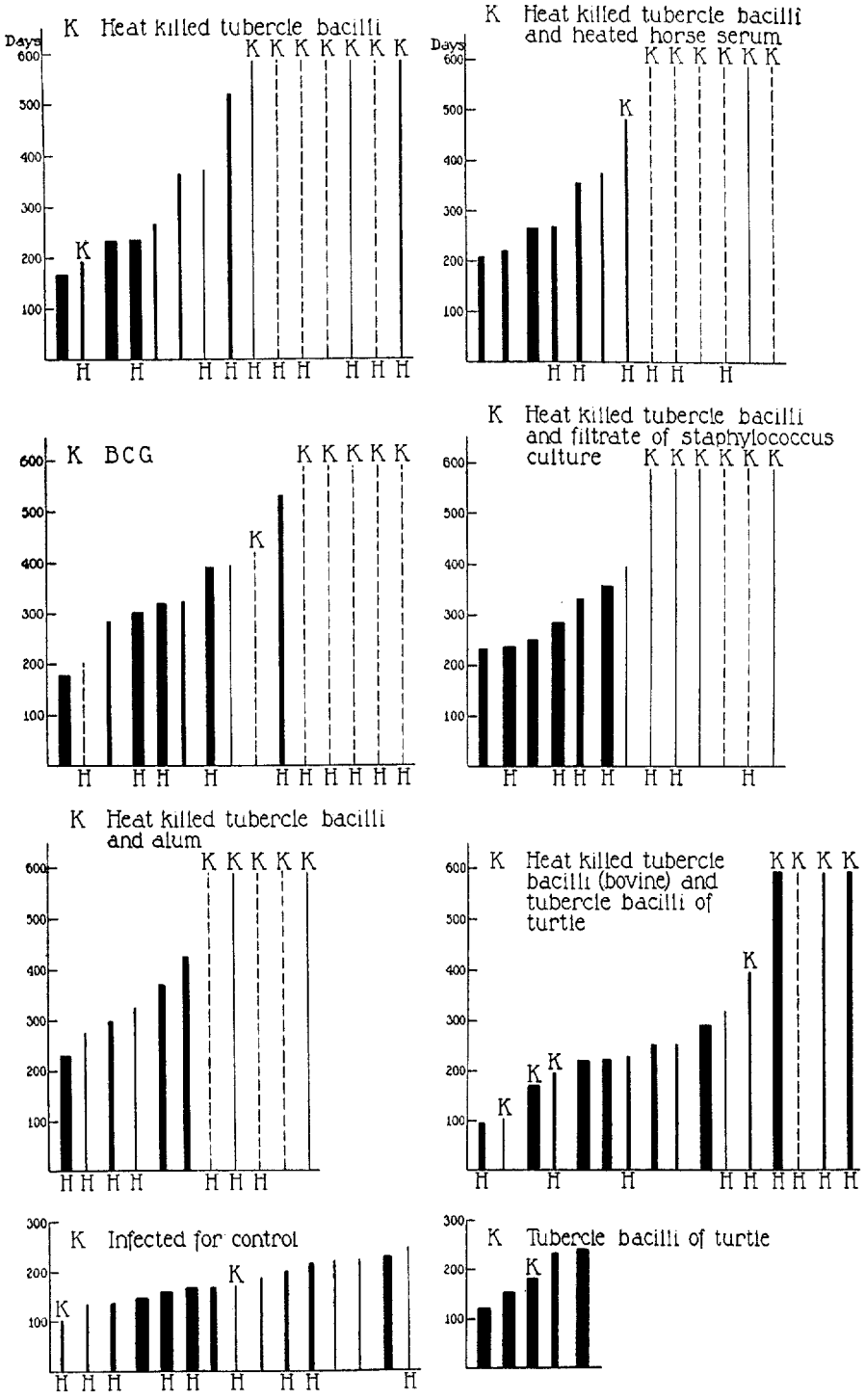

FIG. 4. See description of Experiment K.

and by the same procedure as with group 1, but in all injections were mixed with 0.25 to 0.3 cc. of 0.1 per cent solution of alum.

Group 4. Bovine tubercle bacilli killed by heat were injected in the same doses and by the same procedure as in group 1, and each injection contained 0.2 cc. of a mixture of equal parts of horse serum and 0.5 per cent sodium carbonate heated to 100°C. for a half hour.

Group 5. Bovine tubercle bacilli killed by heat were injected in the same doses and by the same procedure as with group 1, but filtrate from a culture of *Staphylococcus aureus* was added to all injections. The quantity of filtrate added to the first 8 injections was 0.25 cc. and to the remaining injections 0.05 cc.

Group 6. Heat killed tubercle bacilli were injected as with group 5 but to each injection an equal weight of turtle tubercle bacilli was added (see group 7).

Group 7. Turtle tubercle bacilli were given intracutaneously, 0.5 mg. with the first injection, 0.25 mg. with the second, 0.1 mg. with the third and 0.05 mg. with the fourth and subsequent injections.

Infection (see group 8) was intravenous with 0.00001 mg. of bovine tubercle bacilli given 9 days after the last vaccinating injection.

Group	Immunization with	Number of Havana rabbits	Number of Havana rabbits with no tuberculosis	Average length of life after infection of Havana rabbits with tuberculosis	Number of rabbits of other breeds	Number of rabbits of other breeds with no tuberculosis	Average length of life after infection of rabbits of other breeds dying with tuberculosis
				days			*days*
1	Heat killed bovine tubercle bacilli	12	3	510	4	0	404.7
2	BCG	10	4	488.7	5	1	288.2
3	Heat killed bovine tubercle bacilli and alum	7	2	345.8	4	1	460
4	Heat killed bovine tubercle bacilli and heated horse serum	6	3	368.3	7	2	360
5	Heat killed bovine tubercle bacilli and filtrate of staphylococcus culture	7	1	369.5	6	1	429.8
6	Heat killed bovine tubercle bacilli and turtle tubercle bacilli	9	1	427	7	0	214.7
7	Turtle tubercle bacilli				5	0	185.6
8	Infected controls	9	0	170.7	6	0	195.6

In our earlier experiments, as in most of those heretofore undertaken to test resistance of immunized animals to tuberculosis, the infecting dose has been large. Table I shows that a quantity of tubercle bacilli from 0.01 to 0.0001 mg. causes death within an average period of 70.6 to 88.2 days, but with a smaller infecting quantity, namely, 0.00001 mg., of tubercle bacilli, the average duration of life after infection has

increased. It is noteworthy that control animals infected with 0.00001 mg. of bovine tubercle bacilli have died without exception, but when this infecting dose has been decreased tenfold, namely, to a millionth of a milligram, some animals have survived. For the purposes of these experiments the optimal infecting quantity of tubercle bacilli is evidently the least that is consistently fatal.

Infection with Tubercle Bacilli in Different Breeds of Rabbits

The possibility that the Havana breed of rabbits is unusually resistant to tuberculosis has been suggested by observations of Rosahn

TABLE I

Intravenous Infection with Bovine Tubercle Bacilli and the Effect of Immunization with Heat Killed Tubercle Bacilli in Rabbits of Different Breeds and in Havana Rabbits

Animals infected with bovine tubercle bacilli as controls				Animals immunized with heat killed tubercle bacilli and infected with bovine tubercle bacilli			
Number of animals	Infecting dose	Number with no tuberculosis	Average duration of life after infection	Number of animals	Infecting dose	Number with no tuberculosis	Average duration of life after infection
	mg.		*days*		*mg.*		*days*
Various Races of Rabbits							
5	0.01	0	70.6	5	0.01	0	58.4
23	0.001	0	88.2	23	0.001	0	112.3
25	0.0001	0	85.6	49	0.0001	0	131.9
67	0.00001	0	137.3	86	0.00001	8	245.9
Havana Rabbits							
18	0.00001	0	174.3	22	0.00001	6	478.5

(24), who has found that this breed is more resistant to experimental syphilis than other breeds. The number of experiments with Havana rabbits is sufficient to show that control animals infected with 0.00001 mg. of bovine tubercle bacilli live longer and when vaccinated resist the infection more effectively than other breeds which include Chinchilla, Himalayan, Dutch, New Zealand White and mongrel white or brindle-gray American rabbits. With Havana rabbits the frequency of survival after vaccination has been greater than with other breeds, and the average duration of life of those with fatal infection has been longer.

Immunization with Heat Killed Tubercle Bacilli

It is noteworthy that the procedure employed in these experiments is the repeated injection of measured quantities of heat killed tubercle bacilli into the skin or subcutaneous tissue of rabbits, usually made at intervals of from 5 to 7 days. When the injections are intracutaneous the progress of sensitization can be measured by the extent of the inflammatory reaction which occurs at the site of injection and reaches its height within 48 hours. When 0.2 mg. of heat killed tubercle bacilli suspended in 0.2 cc. of salt solution are injected at intervals of 1 week the reaction at the site of injection serves as an index of the progress of sensitization (Fig. 5). The diameter of the inflammatory reaction that follows injection measured after 48 hours increases more or less rapidly and reaches a maximum after from 6 to 10 weeks. At this time edema is more extensive than later, when the area affected is diminished though the elevation is often greater. After about 10 weeks suppuration usually occurs at the site of injection, and there is superficial ulceration followed by healing which may occur promptly or after considerable delay. The changes just described are approximately parallel with sensitization measured by injections of old tuberculin (Fig. 5). The skin reaction to tuberculin increases rapidly, reaches a maximum and then tends to decrease slightly in extent. Fig. 5 shows that healthy rabbits with few if any exceptions are sensitized by repeated injections of heat killed tubercle bacilli. Complement fixation, using heat killed tubercle bacilli as antigen, increases more rapidly than sensitization, reaches a maximum after from 4 to 6 weeks, and then remaining at a high level fluctuates slightly.

The influence of inoculation with heat killed tubercle bacilli on resistance against tuberculous infection is well shown in Table I in which animals from all of the experiments are assembled. The number of animals that have been infected with 0.01 mg. of bovine tubercle bacilli is so small that no satisfactory comparison can be made between "vaccinated" and control animals. It is not improbable that sensitization has hastened the death of these animals infected with 1000 times the fatal dose of tubercle bacilli for the "vaccinated" animals have lived an average of only 58.4 days, whereas the untreated controls lived 70.6 days. When infection has been produced by 0.001

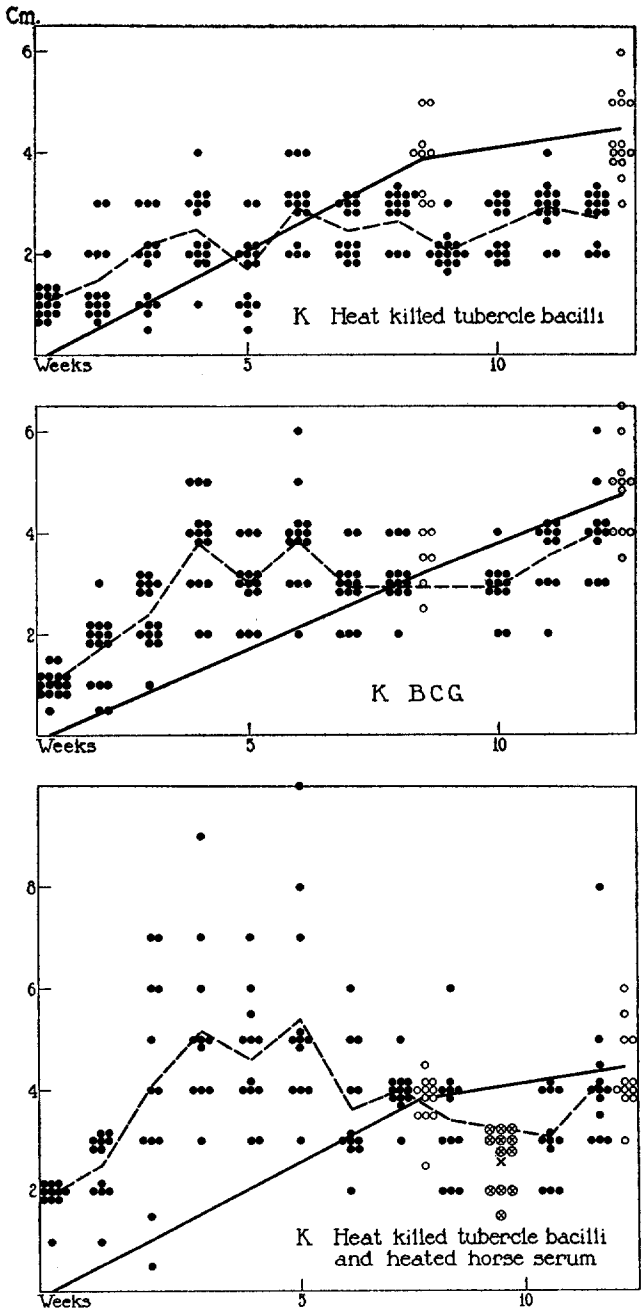

FIG. 5. Progress of sensitization in Experiment K, groups 1, 2 and 4. The reaction to heat killed tubercle bacilli, BCG or heat killed tubercle bacilli with horse serum is shown for each animal by a black dot and the average course of sensitization by a broken line. Sensitization tested by tuberculin is shown by small circles and its average course by a continuous black line. In the animals treated with heat killed tubercle bacilli and horse serum tests were made with heat killed tubercle bacilli alone 9 weeks after the beginning of immunization (indicated by circles with cross, the average being shown by a cross alone).

and 0.0001 mg. of tubercle bacilli the life of treated animals has been greater than that of controls, but none have survived. When rabbits have been infected with 0.00001 mg. of tubercle bacilli approximately 10 per cent have been entirely free from tuberculosis at death and those with tuberculosis have lived nearly twice as long as controls. When Havana rabbits have been used 27 per cent had no tuberculosis at death and the length of life of those that died with some tuberculosis has been nearly three times that of controls. It is noteworthy, moreover, that some of these animals die from intercurrent disease and the extent of tuberculosis is much less than in controls.

The influence of protective inoculation on resistance to tuberculous infection is best shown by experiments in which groups of animals that have received repeated injections of heat killed tubercle bacilli have been infected and subsequently kept under identical conditions with animals that have had no treatment preceding infection.

In Experiment E (Fig. 1) the fate of 25 rabbits immunized with heat killed human tubercle bacilli and 24 "vaccinated" with heat killed bovine tubercle bacilli may be compared with 34 control animals infected with the same suspension of tubercle bacilli and subsequently kept in adjacent cages. Of the vaccinated animals two had no tuberculosis at the time of death and five others had scant involvement of the lungs indicated in the graphs (Fig. 1) by the width of the vertical columns representing the length of life after infection. The greater length of life, recovery from tuberculosis and the less extent of tuberculosis in animals immunized with heat killed tubercle bacilli is even more conspicuous in Experiments G (Fig. 3) and K (Fig. 4) in which Havana rabbits were in large part used.

Immunization with BCG

The attempt has been made to compare the protective action of heat killed tubercle bacilli with that of the attenuated bovine bacillus of Calmette and Guérin (BCG) used under conditions that will produce maximum immunization as measured by the different procedures at our disposal, namely, by sensitization and antibody formation, but chiefly by resistance to infection. BCG in measured quantity was repeatedly injected into the cutis of animals at intervals of 5 to 7 days. In any one experiment the quantity injected and the number of

injections of heat killed tubercle bacilli and of BCG have been the same. The effect of the two agents on sensitization is shown in Fig. 5; their relation to sensitization and antibody formation will be discussed in a later publication.

Comparison of Tables I and II indicates that BCG is somewhat more effective in increasing resistance against tuberculous infection than heat killed tubercle bacilli. The per cent of animals of different races that resisted infection after treatment with BCG (9.3 per cent) was the same as that after administration of heat killed bacilli but the length of life of animals with tuberculosis was greater after the former. When Havana rabbits were used a greater number were

TABLE II

Immunization with BCG

Animals infected with bovine tubercle bacilli as controls				Animals immunized with BCG and infected with bovine tubercle bacilli			
Number of animals	Infecting dose	Number with no tuberculosis	Average duration of life after infection	Number of animals	Infecting dose	Number with no tuberculosis	Average duration of life after infection
	mg.		*days*		*mg.*		*days*
Various Races of Rabbits							
67	0.00001	0	137	43	0.00001	4	337.5
Havana Rabbits							
18	0.00001	0	174.3	20	0.00001	10	447.5

protected by BCG (50 per cent) than by heat killed tubercle bacilli (27 per cent) but the length of life of those with tuberculosis was slightly greater with the latter.

In Experiment E (Fig. 1), in which several breeds of rabbits were used, immunization with heat killed tubercle bacilli and with BCG is compared under identical conditions. Protection as indicated by length of life and extent of tuberculosis (see width of vertical columns in the figure) is considerably greater with BCG, but in Experiments G (Fig. 3) and K (Fig. 4) performed in great part on Havana rabbits little difference is evident, though in both instances the number of animals with no tuberculosis was greater following vaccination with BCG.

Immunization with Heat Killed Tubercle Bacilli and Denatured Serum Protein

Experiments of Lewis and Loomis (25) have shown that immunization with one antigen may accelerate the production of antibodies by another. They found that production of hemolytic amboceptors for erythrocytes of sheep proceeded more rapidly in tuberculous than in normal animals. In the experiments that will be described some increase of the resistance to infection with virulent tubercle bacilli has been obtained by injecting heat killed tubercle bacilli in combination with another antigen, such as heated serum of horse or of sheep (Table III). The course of sensitization with this procedure is shown in Fig. 5.

TABLE III

Immunization with Heat Killed Tubercle Bacilli and Serum Proteins

	Infecting dose	Number of animals	Number with no tuberculosis	Average duration of life of animals with tuberculosis
	mg.			*days*
Various Races of Rabbits				
Heat killed tubercle bacilli and native horse serum	0.00001	12	0	183.1
Heat killed tubercle bacilli and heated horse serum	0.00001	30	4	
Heat killed tubercle bacilli and heated sheep serum	0.00001	15	1	245.5+
Controls	0.00001	67	0	137.3
Havana Rabbits				
Heat killed tubercle bacilli and heated horse serum	0.00001	6	3	368.3+
Controls	0.00001	18	0	174.3

Unheated horse serum combined with heat killed tubercle bacilli has diminished the resistance conferred by heat killed tubercle bacilli so much that resistance to infection of animals treated with the two agents is only slightly, if at all, greater, than that of controls. Rabbits of various breeds treated with heated horse serum or heated sheep serum in combination with killed tubercle bacilli have shown somewhat greater resistance than animals that have received heat killed tubercle bacilli alone (compared with Table I). The serum was diluted with an equal part of 0.5 per cent solution of sodium carbonate

and maintained at 100°C. for half an hour. One-half of the small number of Havana rabbits that have been treated with heated horse serum and killed tubercle bacilli have successfully resisted infection, and in these animals resistance seems to have been considerably greater than that with heat killed tubercle bacilli alone.

The association of heated horse serum with heat killed tubercle bacilli has increased the protective action of the latter and has made it equal to that of BCG. This relation is well seen in Experiment E (Fig. 2) performed on several different breeds of rabbits, and in Experiment K (Fig. 4) performed on Havana rabbits; in both experiments the duration of life and extent of tuberculous lesions has been almost the same in animals immunized with heat killed tubercle bacilli and in those immunized with BCG.

Immunization with Heat Killed Tubercle Bacilli in Association with Some Bacterial Antigens

The bacterial antigens, or groups of antigens, contained in heat killed typhoid bacilli and in filtrates from cultures of *Staphylococcus aureus* have been combined with heat killed tubercle bacilli in order to determine if the immunizing activity of the latter is increased by one or other of them. Either commercial typhoid vaccine (Experiment C, group 6; Experiment D, group 5, and Experiment E, group 6) or typhoid bacilli grown in the laboratory and killed by heat at 60°C. for 45 minutes (Experiment F, group 3) was used. Staphylococcus filtrate was prepared from cultures of *Staphylococcus aureus* in bouillon grown 4 days and was preserved by addition of 0.35 per cent tricresol.

Animals that have received typhoid bacilli alone survive infection during approximately the same time as controls and no increase of the immunizing power of heat killed tubercle bacilli has been produced by the addition of heat killed typhoid bacilli. In experiments on a small number of rabbits addition of filtrate from cultures of *Staphylococcus aureus* has apparently influenced favorably the course of immunization produced by heat killed tubercle bacilli but the data are not significant and when Havana rabbits were used protection was less with the associated antigens than with heat killed tubercle bacilli alone.

Immunization with Heat Killed Tubercle Bacilli in Association with Some Antigens Derived from Living Acid-Fast Bacilli

Experiments were undertaken to determine if defects in the antigens of tubercle bacilli killed by heat can be replaced by certain unheated derivatives of the bacterium, namely, by filtrates from cultures of the bovine tubercle bacillus or by addition of a related acid-fast microorganism that is non-pathogenic for rabbits. For the latter purpose the turtle bacillus of Friedmann (26) was chosen because it

TABLE IV

Immunization with Heat Killed Tubercle Bacilli in Association with Bacterial Antigens

	Infecting dose	Number of animals	Number with no tuberculosis	Average duration of life of animals with tuberculosis
	mg.			*days*
Various Races of Rabbits				
Heat killed tubercle bacilli	0.00001	86	8	245.9
Heat killed tubercle bacilli and heat killed typhoid bacilli	0.00001	35	1	245.5
Heat killed typhoid bacilli	0.00001	13	0	127.7
Heat killed tubercle bacilli and filtrate of staphylococcus culture	0.00001	6	1	429.8
Controls	0.00001	67	0	137.3
Havana Rabbits				
Heat killed tubercle bacilli	0.00001	22	6	478.5
Heat killed tubercle bacilli and filtrate of staphylococcus culture	0.00001	7	1	369.5+
Controls	0.00001	18	0	174.3

apparently produces some increase of resistance to infection with human and bovine tubercle bacilli. The filtrate was prepared from a bouillon culture of tubercle bacilli 6 weeks old; it was passed through a Seitz filter and concentrated at 42–45°C. *in vacuo* to one-fourth of its volume.

Filtrate of cultures of tubercle bacilli used alone as an immunizing agent both in Havana and in other rabbits has increased the length of life after infection as compared with infected controls but has in no instance inhibited the development of tuberculosis (Experiment G).

TABLE V

Immunization with Heat Killed Tubercle Bacilli and Unheated Filtrate from Culture of Tubercle Bacilli or with Heat Killed Tubercle Bacilli and Living Acid-Fast Bacilli

	Infecting dose	Number of animals	Number with no tuberculosis	Average duration of life of animals with tuberculosis
	mg.			*days*
Various Races of Rabbits				
Heat killed tubercle bacilli	0.00001	35	1	245
Heat killed tubercle bacilli and filtrate of culture of tubercle bacilli	0.00001	4	0	191
Filtrate of culture of tubercle bacilli	0.00001	4	0	183.2
Heated tubercle bacilli and living turtle tubercle bacilli	0.00001	12	0	206.2
Living turtle bacilli	0.00001	5	0	185.6
Controls	0.00001	67	0	137.3
Havana Rabbits				
Heat killed tubercle bacilli	0.00001	22	6	478.5
Heated tubercle bacilli and filtrate of cultures of tubercle bacilli	0.00001	12	3	494.4
Filtrate of cultures of tubercle bacilli	0.00001	11	0	285.8
Heated tubercle bacilli and living turtle tubercle bacilli	0.00001	8	1	427+
Controls	0.00001	18	0	174.3

TABLE VI

Immunization with Heat Killed Tubercle Bacilli in Association with Alum

	Infecting dose	Number of animals	Number with no tuberculosis	Average duration of life of animals with tuberculosis
	mg.			*days*
Various Races of Rabbits				
Heat killed tubercle bacilli	0.00001	86	8	245.9
Heated tubercle bacilli and alum	0.00001	7	1	339.7
Controls	0.00001	67	0	137.3
Havana Rabbits				
Heat killed tubercle bacilli	0.00001	22	6	478.5
Heated tubercle bacilli and alum	0.00001	7	2	345.8+
Controls	0.00001	18	0	174.3

When the filtrate has been added to heat killed tubercle bacilli (Experiment G) no significant increase of the protection conferred by the dead bacilli has been observed. The same statements concerning the relation of the turtle bacillus of Friedmann (Experiment K, Fig. 4) to heat killed bovine tubercle bacilli can be made. Neither of these agents supplies an antigenic fraction capable of increasing the immunizing power of heat killed tubercle bacilli.

Immunization with Heat Killed Tubercle Bacilli in Association with an Inflammatory Irritant (Alum)

The attempt has been made to increase the immunizing power of heat killed tubercle bacilli by injecting with them a substance, namely, alum, (Experiment K, Fig. 4) that increases the inflammatory reaction produced by heat killed tubercle bacilli alone. The immunizing power of heat killed tubercle bacilli has been diminished rather than increased by the addition of alum.

DISCUSSION

The foremost purpose of these experiments has been to determine if dead tubercle bacilli can be used to replace the living attenuated microorganism (BCG) as a means of protection against tuberculous infection. Widespread use of the BCG of Calmette and Guérin has shown that it sensitizes to products of the tubercle bacillus and confers a considerable measure of protection when administered by intracutaneous or subcutaneous injection.

The original claim of Calmette that BCG failed to produce tuberculous lesions has not been confirmed; a great quantity (20 mg.) given intravenously to rabbits produces fatal pneumonia and smaller quantities administered by subcutaneous injection produce in spleen, liver and lungs true tubercles that heal spontaneously. Administered to infants by mouth, as recommended by Calmette, BCG has uncertain sensitizing activity and protection is doubtful. There is no convincing evidence that BCG cultured by the procedure recommended by Calmette (22) increases in virulence or produces progressive tuberculosis. Nevertheless, suppuration often occurs at the site of injection and "cold abscesses" in regional lymph nodes are not infrequent.

Reviewing the records of 2000 children in Paris to whom BCG was given subcutaneously, Kayne (27) found nodules or abscesses at the site of injection in 50

per cent of those vaccinated between 1928 and 1930, and in only 4 per cent in those similarly treated in 1931 and 1932. He believed that the scant virulence of BCG had diminished during continued cultivation.

Injection of BCG into the cutaneous tissue, Keresturi, Rosenberg and Park (28) found, produced sensitization to tuberculin in 80 per cent of children, and into the subcutaneous tissue in 60 per cent. Sensitization reached a maximum at the end of 6 months and later diminished rapidly so that most children no longer reacted after 2 years. Intracutaneous "vaccination" produced necrosis of the skin in 53 per cent of children and after subcutaneous vaccination in 54 per cent. When injection was made intracutaneously into the thigh, there was suppuration of the inguinal lymph nodes in 15 per cent of children and when injection was subcutaneous in 2 per cent. The optimum quantity of BCG introduced into the skin measured by the tuberculin test was 0.15 mg., but this dose injected into the skin of the thigh caused suppuration of the inguinal lymph nodes in 25 per cent of children.

Earlier experiments have given little support to the opinion that significant immunity can be produced by dead tubercle bacilli. Those who have been most successful in sensitizing animals to tuberculin by injections of heat killed tubercle bacilli have not obtained by the same means effective immunity against infection.

Our experiments on rabbits indicate that heat killed tubercle bacilli cause increased resistance to infection with virulent tubercle bacilli only slightly less than that produced by BCG. This "vaccine," prepared with all of the precautions long practiced in the preparation of typhoid and other bacterial vaccines, eliminates the dangers necessarily incident to the introduction of living bacteria into the body. The immunity that follows injection of heat killed tubercle bacilli, like all immunity against tuberculosis, is relative because it can be overcome by increasing the number of infecting microorganisms, and is perhaps temporary because it is well known that evidence of immunization, notably skin sensitization, against tuberculosis tends to diminish or disappear after 1 or 2 years. Within these limitations it has seemed probable that immunization of susceptible persons exposed to tuberculous infection might be sufficient to influence favorably the course of tuberculous infection. Persons exposed to tuberculosis have been given heat killed tubercle bacilli and it has been possible to obtain evidence in favor of this opinion.

CONCLUSIONS

Heat killed tubercle bacilli repeatedly injected into or below the skin of rabbits increase conspicuously their resistance against infection with virulent tubercle bacilli.

Protection against tuberculous infection following the administration of heat killed tubercle bacilli to rabbits is only slightly less than that given by BCG.

Addition of certain antigens, notably heated horse serum, increases the protection given by heat killed tubercle bacilli so that it is approximately the same as that afforded by BCG.

These experiments and tentative observations of persons exposed to tuberculous infection indicate that heat killed tubercle bacilli may be substituted for the living attenuated microorganism in the attempt to increase resistance against tuberculous infection and to influence favorably the delicate balance between asymptomatic or latent infection and progressive manifest disease that is characteristic of human tuberculosis.

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