Vaccination of guinea-pigs against tuberculosis with B.C.G / C.C. Okell and H.J. Parish.

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VACCINATION OF GUINEA-PIGS AGAINST TUBERCULOSIS WITH B.C.G.

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From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

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VACCINATION OF GUINEA-PIGS AGAINST TUBERCULOSIS WITH B.C.G.

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THIS communication deals with four experiments which have been carried out during the last three years in an endeavour to repeat Prof. Calmette's work on the prophylaxis of tuberculosis in guinea-pigs with vaccine B.C.G. In each experiment a group of animals received one or more injections of the vaccine subcutaneously or intravenously; after intervals of from 5 to 12 weeks to allow for the development of immunity, the vaccinated guinea-pigs and an approximately equal number of controls of similar weights were given an infecting dose of virulent *B. tuberculosis*. All the animals were observed regularly and weighed at weekly or fortnightly intervals. Post-mortem examinations were performed on all dead guinea-pigs. Those animals which died from intercurrent diseases too early in the experiment to develop tubercular lesions visible at autopsy as a result of the infecting dose were excluded from the comparison of the protected and unprotected groups.

Experiment 1.—This experiment is to be regarded as a preliminary one only (Chart 1). Five guinea-pigs, which had received 20 mgm. of B.C.G. subcutaneously 12 weeks previously, were given an infecting dose of virulent organisms (human strain) contained in a suspension of triturated gland from a tubercular guinea-pig. Six guinea-pigs were injected with gland only as controls, and were all dead from generalized tuberculosis at the end of 16th week, the average period of survival being 11 weeks. When the last of the control guinea-pigs died, all the vaccinated guinea-pigs, apparently fit and well-nourished, were killed; at autopsy they all showed evidence of generalized tuberculosis, though the lesions they exhibited were less advanced than in the control animals which had died at an earlier date as a result of their infection.

In later experiments great care was taken to select guinea-pigs of exactly comparable weight and physical condition for the two groups, but in the preliminary experiment the vaccinated animals were in rather better condition and slightly heavier than the controls at the time of infection with virulent organisms. Even when this was taken into consideration the results were of such promise that further experiments were arranged.

Experiment 2.—In this experiment the vaccinated guinea-pigs were given the same dose of B.C.G. (20 mgm.) as in the previous experiment, but the infecting dose was 0·5 mgm. of the virulent human strain we use for the routine infection of guinea-pigs for the standardization of tuberculin. The interval between the injection of the living vaccine and the injection of

virulent culture was 11 weeks. The period of survival of 10 vaccinated and 15 control guinea-pigs is represented in Chart 2. There were 3 survivors in the immunized group, which were killed in the 20th week and were found to be well infected with tuberculosis. If these animals had died from tuberculosis in the 20th week the average period of survival of the vaccinated animals would have been 17 weeks, or 4 weeks longer than that of the controls.

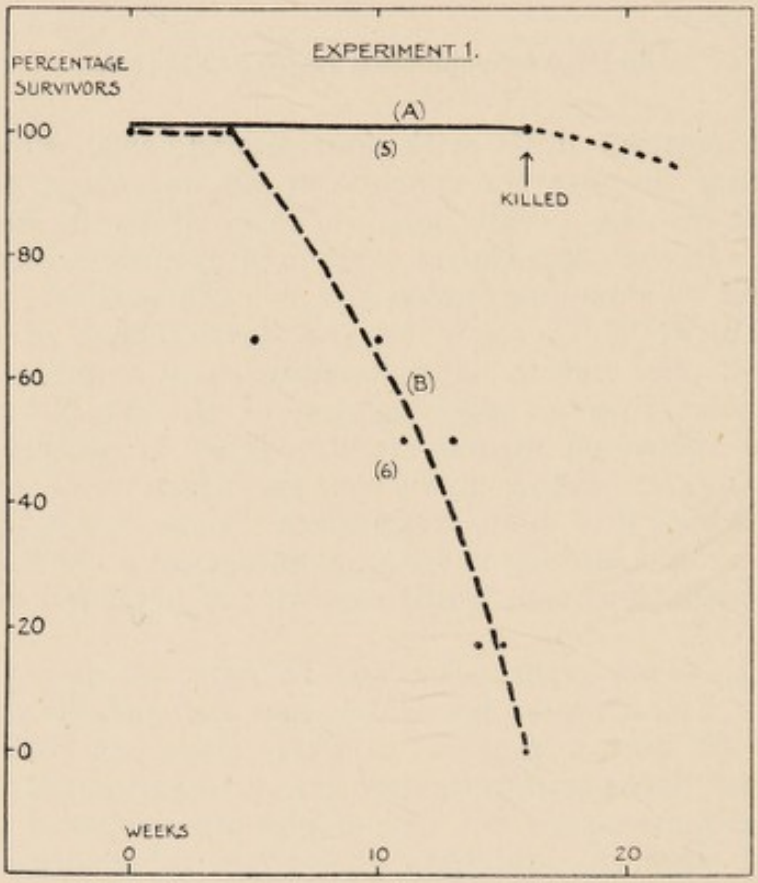

CHART 1.

EXPERIMENT 1.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

A. *Vaccinated guinea-pigs* (——————).
Injected subcutaneously with 20 mgm. B.C.G. After 12 weeks injected subcutaneously with 0·25 c.c. suspension of gland from guinea-pig infected with virulent human strain of *Bacillus tuberculosis*.
Average period of survival, over 16 weeks.

B. *Control guinea-pigs* (— — — — — — —).
Injected with guinea-pig gland only.
Average period of survival, 11 weeks.

Experiment 3.—An attempt was made in this experiment to obtain a greater difference in the period of survival of the two groups by giving five injections of 20 mgm. each of B.C.G. subcutaneously, instead of one only as in previous experiments, and by reducing the infecting dose of virulent culture to 0·25 mgm. The difference between the vaccinated and unvaccinated

guinea-pigs in this experiment did not, however, prove any greater than in similar groups in Experiment 2.

The interval between the last injection of B.C.G. and the injection of virulent culture was 5 weeks. Twenty-one guinea-pigs in the vaccinated group lived on an average just under 7 weeks, or approximately 2 weeks longer than 25 controls (Chart 3).

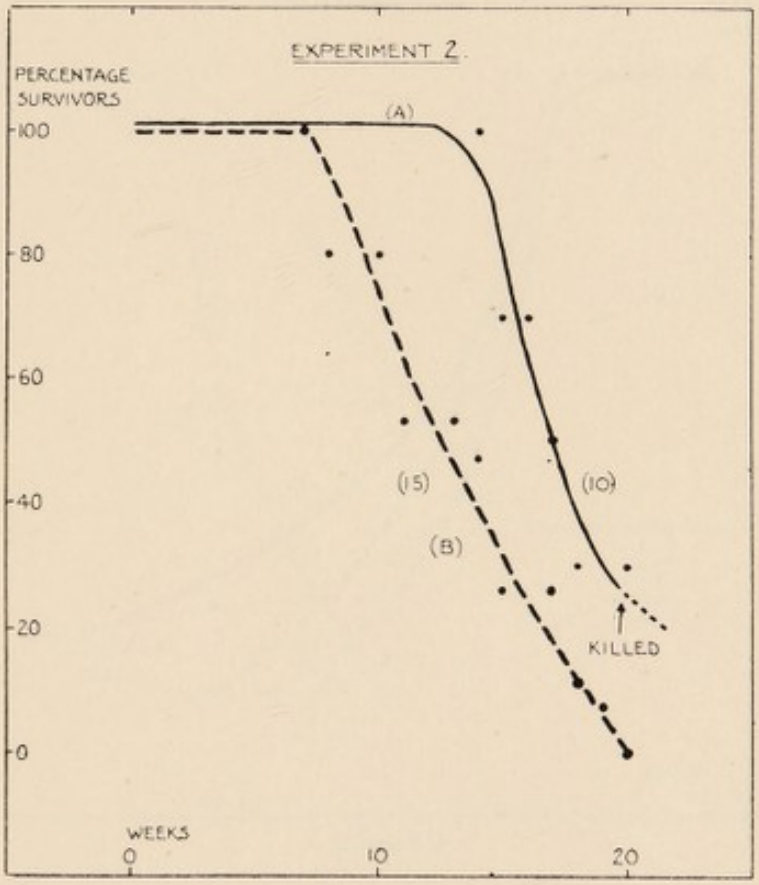

CHART 2.

EXPERIMENT 2.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

A. *Vaccinated guinea-pigs* (————————).
Injected subcutaneously with 20 mgm. B.C.G. After 11 weeks injected subcutaneously with 0·5 mgm. of a virulent human strain of *Bacillus tuberculosis.*
Average period of survival, over 17 weeks.

B. *Control guinea-pigs* (— — — — — — —).
Injected with virulent culture only.
Average period of survival, 13 weeks.

The average period of survival of a small group of 13 guinea-pigs which had received multiple injections of Koch's bacillary emulsion (B.E.) was only one week longer than that of the controls. One cannot draw conclusions from an experiment on so small a number of animals, but the result is suggestive, and lends support to the observations of other workers on the immunity produced by B.E., dead tubercle bacilli and many allied vaccines.

Experiment 4.—This experiment was arranged as far as possible on the lines recommended by Prof. Calmette, and, by choosing a critical infecting

dose, we hoped to exaggerate the differences between the control and vaccinated guinea-pigs which were evident in the previous experiments. A single injection of the vaccine B.C.G. was given intravenously, followed after an interval of 5 weeks by 0·01 mgm. of the virulent bovine strain used by Prof. Calmette

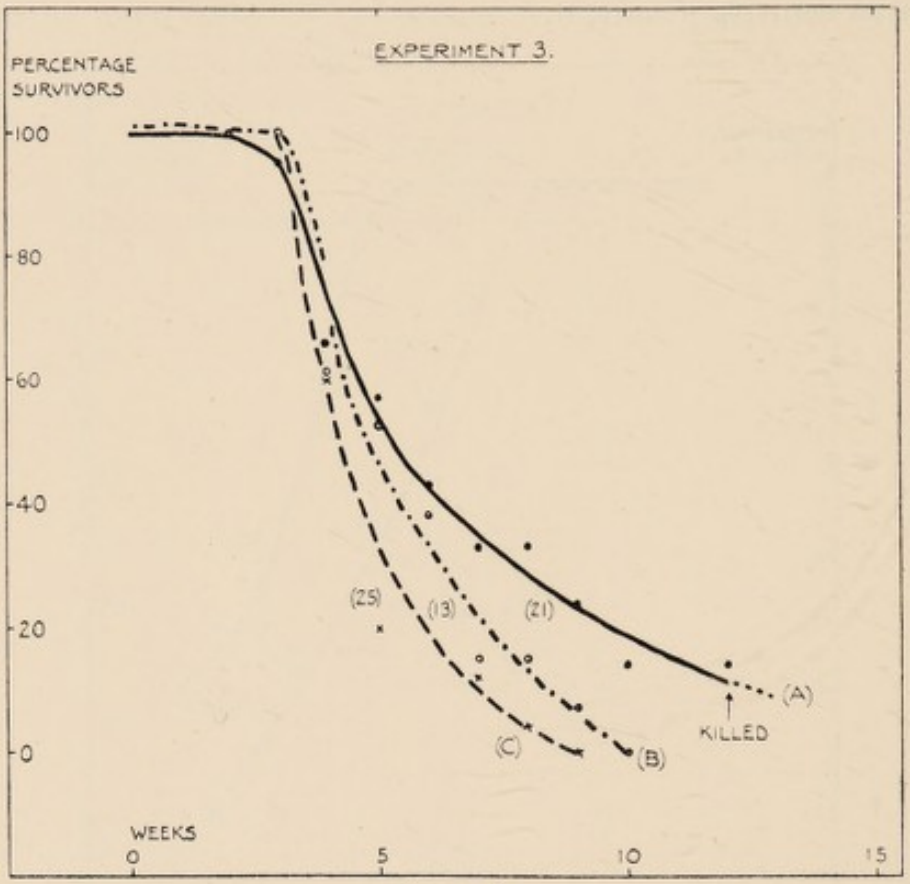

CHART 3.

EXPERIMENT 3.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

Vaccinated guinea-pigs.

(A) (——————). Received subcutaneously 5 injections of 20 mgm. each of B.C.G. in 9 weeks. After 5 weeks injected subcutaneously with 0·25 mgm. of a virulent human strain of *Bacillus tuberculosis.*

Average period of survival, 7 weeks.

(B) (— · — · — · —). Received subcutaneously 5 injections of 20 mgm. each of B.E. in 9 weeks. After 5 weeks injected subcutaneously with 0·25 mgm. of a virulent human strain of *Bacillus tuberculosis.*

Average period of survival, 6 weeks.

Control guinea-pigs (— — — — — — —).

(C) Injected with virulent culture only.

Average period of survival, 5 weeks.

in similar experiments. A 3 weeks' growth of the virulent culture from potato medium was ground in sterile 0·9% saline solution in an agate mortar, and standardized to contain 1 mgm. of tubercle bacilli per c.c. The unfiltered suspension, which was homogeneous to the naked eye and showed only isolated bacilli or small clumps of acid-fast organisms microscopically, was thoroughly

shaken before being used. The infecting dose was 0·01 c.c. (containing 0·01 mgm.), which was placed on the eye-ball with the aid of Trevan's micrometer syringe. Using this syringe and such small amounts, the risk of loss from overflowing on to the cheek would appear to be a negligible one,

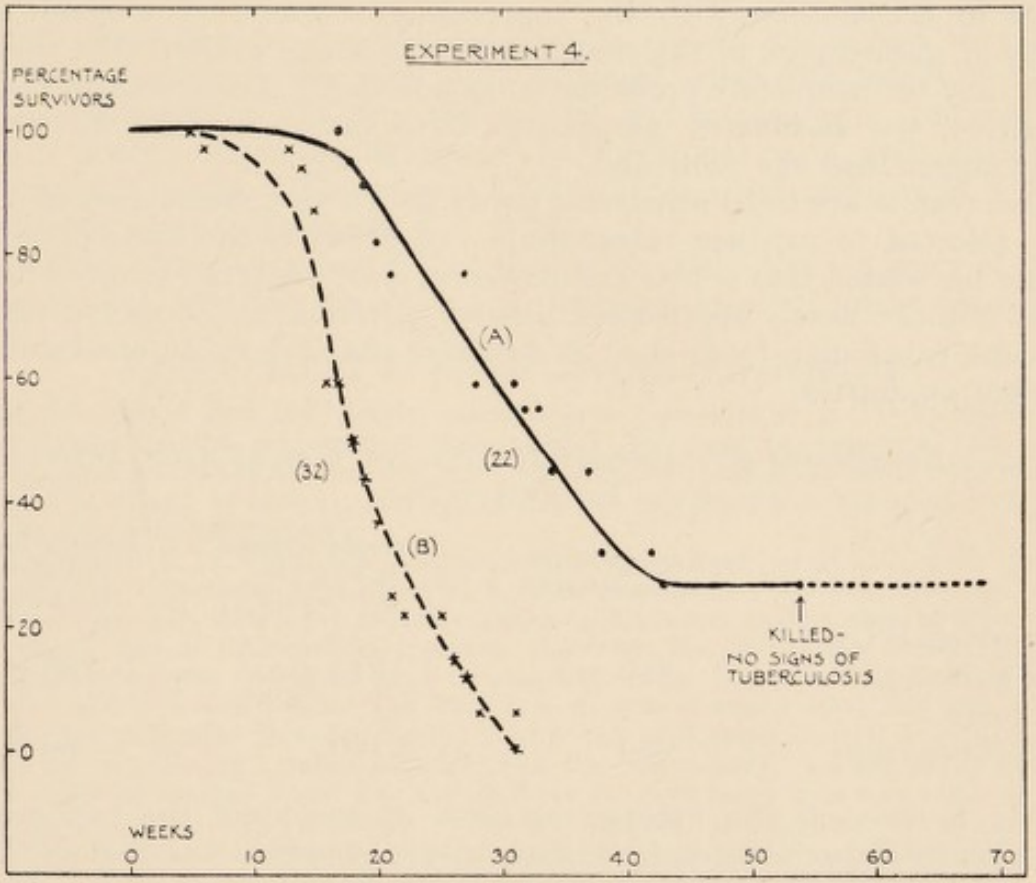

CHART 4.

EXPERIMENT 4.

Figures in brackets indicate number of guinea-pigs under experiment in each group.

A. *Vaccinated guinea-pigs* (——————————).
Injected intravenously with 20 mgm. B.C.G. After 5 weeks 0·01 mgm. of a virulent bovine strain (Calmette) of *Bacillus tuberculosis* placed on the eye-ball.
Average period of survival, over 35 weeks, or, excluding 6 tubercle-free survivors, 29 weeks.

B. *Control guinea-pigs* (— — — — — — —).
Injected with virulent culture only.
Average period of survival, 20 weeks.

and although only a small drop was instilled, many tubercle bacilli must have come in contact with each animal's conjunctiva. In a preliminary experiment 0·01 mgm., of virulent culture placed on the eye-ball infected 9 of 10 guinea-pigs (the tenth animal lived for 10 months and post-mortem showed no signs of infection); 0·0001 mgm., the dose recommended by Prof. Calmette, produced only a few lesions in the lungs of 1 of 10 guinea-pigs. It was

therefore decided that 0·01 mgm. would probably represent a satisfactory " minimal infecting dose " for Experiment 4.

The average period of survival of 32 control guinea-pigs in this experiment (Chart 4) was 20 weeks. In the vaccinated group of 22 guinea-pigs, 6 showed no signs of tuberculosis when killed in the 54th week. When this result is taken in conjunction with the much longer period of survival of the majority of guinea-pigs in the control group, it seems evident that vaccinated animals may be completely protected from infection. Even if these 6 animals are omitted, the vaccinated guinea-pigs lived on an average 29 weeks, or 9 weeks longer than the controls.

These results are to be attributed partly to the very small dose of virulent culture selected to produce tuberculosis, and partly to the route of infection. Calmette has stated that ocular instillation is to be preferred to other methods, because it more nearly approaches natural infection, and does not produce a permanent tubercular focus such as develops after the subcutaneous injection of virulent organisms.

TABLE I.—*Experiment 4: Average Increase in Weight after Infection with Virulent T.B.*

	Average weight when infected.	Average increase in weight— 0–10 weeks.	Average increase in weight— 0–15 weeks.
Vaccinated B.C.G.:			
22 guinea-pigs	360 gm.	210 gm.	180 gm.
Controls:			
32 guinea-pigs	345 „	140 „	68 „

Table I, showing the average increase in weight of the two groups of guinea-pigs in Experiment 4, lends support to the conclusion that B.C.G. has a restraining influence on the development of tuberculosis in the guinea-pig.

The results obtained in Experiment 4, where a critical infecting dose has been used, are very striking, and numerical analysis indicates that they are significantly in favour of a definite, though by no means complete, immunity to tuberculosis.

In Experiments 2, 3 and 4 there has been no difference in the post-mortem appearances of guinea-pigs belonging to either the vaccinated or the control groups which have died from tuberculosis. Enlargement of the cervical glands was apparent at autopsy in Experiment 4, in which the guinea-pigs were infected by the ocular route.

B.C.G. Does Not Cause Tuberculosis in Guinea-Pigs.

B.C.G. has never seemed to produce any of the lesions of tuberculosis during the period of observation of these experiments. In each experiment 6 or more vaccinated guinea-pigs which have not received an injection of virulent organisms subsequently have remained well for several months, and have shown no signs of tubercular infection when killed. It should be pointed out, however, that Petroff, Branch and Steenken (1927) state they were able to obtain virulent " variants " from cultures of B.C.G.

One of the guinea-pigs in the vaccinated group in Experiment 4 which died shortly before it was due to receive an infecting dose of virulent organisms showed at the autopsy several pulmonary, hepatic and splenic lesions which resembled tubercular granulomata, but were non-infective for 4 other guinea-pigs. A few acid-fast bacilli were seen after prolonged search of a smear prepared from the spleen, and an organism of the Salmonella group was obtained on culture from the same organ. Calmette (1927) has stated that similar lesions may sometimes be seen 3 to 5 weeks after the intravenous injection of large doses of B.C.G. into rabbits and guinea-pigs, and are to be ascribed to emboli of the capillaries and to the formation of giant-cells around the clumps of bacilli. They never progress to calcification, and disappear spontaneously after some weeks.

It may be added in passing that B.C.G. renders the majority of guinea-pigs slightly but definitely sensitive to tuberculin, injected intracutaneously.

Effect of B.C.G. on General Health of Guinea-pigs.

In Experiments 1 and 2 a single subcutaneous injection of B.C.G. produced small abscesses locally in the majority of guinea-pigs. In these experiments, and again in Experiment 4, in which the vaccine was given intravenously, the death-rate of the vaccinated animals over a period of several weeks following the injection was not appreciably higher than of the uninoculated controls.

In Experiment 3, in which five injections of 20 mgm. each of B.C.G. were given subcutaneously, the injections were given at first at weekly intervals but were not tolerated very well; the animals developed extensive abscess-formation at the sites of injection, and many of them died of intercurrent diseases. Towards the end of the course the interval between injections was increased to 3 weeks, but when the infecting dose of virulent culture was given 14 weeks after the first injection or 5 weeks after the last, 25 of the 50 guinea-pigs originally included in the group had died, representing a 50% mortality. Another 4 animals died of a Salmonella infection a few days later. In this group of animals, therefore, 58% of deaths were not attributable to tubercular infection and have been excluded from Chart 3. The mortality in the control group in the same period was 37%.

In all four experiments deaths not attributable to tuberculosis are not included in the charts, but, in order to give the full experimental results, are recorded in Table II.

TABLE II.—*Deaths* not *attributable to Tuberculous Infection and excluded from the Charts.*

Number of experiment.	Original number of guinea-pigs.		Dose of B.C.G. in mgms.	From date of *first* injection of B.C.G. to time of injection with virulent T.B.		After virulent T.B. injected.	Deaths not attributable to tuberculosis.	
	Vaccinated with B.C.G.	Controls.		Weeks.	Deaths.	Deaths.	Total number.	Percentage.
1	6	...	20	12	0	1	1	16
	...	6	0	12	0	0	0	0
2	20	...	20	11	8	2	10	50
	...	26	0	11	9	2	11	42
3	50	...	100*	14	25	4	29	58†
	...	40	0	14	12	3	15	37
4	35	...	20	5	8	5	13	37
	...	45	0	5	7	6	13	28

* Five successive doses of 20 mgm. in 9 weeks.

† In a similar experiment with Koch's bacillary emulsion (B.E.), 57% of deaths were not attributable to tuberculous infection.

Statistical Note.

We are indebted to Dr. J. W. Trevan for the following statistical note:

Experiment 1.—The mean time of survival for the vaccinated guinea-pigs was obtained by taking the time at which the five were killed as the survival time for those five. Tested by the second method given by Fisher ('Statistical Methods for Research Workers,' p. 109), the value of t obtained is 2·08, which corresponds to a value of $P < 0{\cdot}1$ and $> 0{\cdot}05$. The mean times of survival are therefore not significantly greater for the vaccinated guinea-gigs; but if the vaccinated guinea-pigs had all lived for 18 weeks, as they probably would have done, the result would have been significant.

Experiment 2.—The value of t obtained in the same way is 3·08 ($P < 0{\cdot}01$), which indicates that there is a high degree of probability that the difference in survival time is statistically significant.

Experiment 3.—For B.C.G. injected guinea-pigs and controls $t = 2{\cdot}22$ ($P < 0{\cdot}05 > 0{\cdot}02$); the difference of survival time of the vaccinated and of the control animals is therefore significant. For B.E. injected guinea-pigs and controls $t = 1{\cdot}25$ ($P = 0{\cdot}2$); the difference is therefore not significant.

Experiment 4.—Of the guinea-pigs in this experiment 6 of the vaccinated were found free from tuberculosis when killed. None of the other controls failed to show tuberculous lesions. For an event which shows 6 successes out of 22 trials the standard deviation is $\sqrt{\frac{6}{22} \times \frac{16}{22}} = 2{\cdot}09$, and the chance against none dying in 32 similar animals injected at the same time is therefore about 300 to 1. If we also use as controls the previous group of 10 unvaccinated guinea-pigs of which 1 failed to become infected the difference is still significant. There is therefore statistically significant evidence that B.C.G. vaccinated guinea-pigs may be completely protected from infection.

The remaining 16 vaccinated guinea-pigs had an average survival time of 28·6 weeks; that of the controls was 19·7 weeks. The value of t is 4·27 ($P < 0{\cdot}01$); the mean survival time of the vaccinated guinea-pigs is significantly greater than that of the controls.

DISCUSSION.

From these experiments it seems clear that by means of B.C.G. guinea-pigs can be partially and in some cases completely protected from infection with a dose of virulent bacilli. The results seem to be worth recording in detail, because there are so many conflicting views in the literature as to the efficacy of any form of tuberculosis vaccine. We believe that the evidence recorded here proves beyond all reasonable doubt that a vaccine which in our hands at least was apparently harmless has given rise to a significant immunity.

From the time of Koch's original experiment showing the modified reaction of an animal to a second dose of virulent bacilli, much evidence has been produced that cultures, attenuated or having a low virulence to the particular animal under test, are able to produce some degree of immunity (*e.g.* Krause, 1926). Frequently the evidence in favour of these experiments has been submitted to much destructive criticism, as in the case of Von Behring's bovo-vaccine.

One has, of course, no guarantee that B.C.G. represents a permanently avirulent strain. Indeed, the recent work of Petroff and his colleagues (1927) suggests that it is capable of dangerous reversion. In our experiments, at least, it has never infected guinea-pigs, and therefore represents a move in the right direction as compared with the partially virulent strains with which most of the sound work of the past has been done.

It is difficult to judge from the literature whether the effect demonstrated here in the case of B.C.G. could be paralleled by vaccines of dead tubercle bacilli, either intact or ground up in the form of B.E., or even by old

tuberculins. Many of these preparations were stated to have been able to prolong the life of guinea-pigs.

Zinsser, Ward and Jennings (1925), using dead tubercle bacilli as a vaccine, kept treated guinea-pigs alive for much longer periods than the controls, although apparently they did not arrange an experiment with an infecting dose sufficiently "critical" to get complete protection.

It seems quite definite that in experiments with guinea-pigs such as we have described, some measurable protection is conferred on the vaccinated animals. Calmette describes a much higher degree of protection in cattle and apes, but as our experiments did not relate to these animals such considerations were outside the scope of the present paper.

SUMMARY AND CONCLUSIONS.

(1) A living vaccine of Calmette and Guérin's strain of *B. tuberculosis* (B.C.G.) has not caused tuberculosis in guinea-pigs even after an intensive course of 5 doses of 20 mgm. each of the living vaccine.

(2) Guinea-pigs inoculated with B.C.G. and later with virulent *B. tuberculosis* (human or bovine strains) tend to live for longer periods than others which have received an infecting dose of virulent organisms only. This is particularly marked in Experiment 4, where the infecting dose was minimal; in this experiment complete protection was obtained in 27 % of animals.

(3) The vaccinated guinea-pigs either showed no recognizable signs of tubercular infection as in the case of 6 guinea-pigs in Experiment 4, or, in the case of guinea-pigs which died from the disease, the infection was generalized and similar to that of the controls.

(4) It is uncertain from the evidence here produced that B.C.G. is a much better experimental prophylactic than living attenuated bacilli, Koch's bacillary emulsion (B.E.), dead tubercle bacilli, or certain other vaccines which have been in vogue at various periods in the past.

We have to express our thanks to Prof. Calmette for his constant kindness throughout this work, in supplying the original cultures and in allowing us to consult him concerning the details of his experiments.

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