STUDIES ON B. C. G. *

II. VACCINATION OF GUINEA PIGS

RICHARD THOMPSON and ENRIQUE KÖPPISCH
From the Departments of Bacteriology and Pathology, School of Tropical Medicine, San Juan, Puerto Rico.

Many workers who have attempted to immunize laboratory animals against virulent tubercle bacillus infection by vaccination with B.C.G. have found no evidence of protection. Others have succeeded to some degree, but in no case has this protection been very marked; usually it has been evident only when minimal reinfective doses were used. We cannot attempt to give even a sketchy summary of the great volume of literature on this topic, but shall refer only to certain publications which we consider as having direct bearing on the experiments reported here. Complete summaries are included in the reviews mentioned in the previous paper [1, 2, 3, 4, 5 and 6].

The majority of the experiments to be reported are concerned with the vaccination of new-born guinea pigs by the administration of B.C.G. cultures by mouth. Calmette, Nègre and Boquet [7] and Tzeknowitzer [8] obtained a slight degree of protection when guinea pigs were given B.C.G. by mouth and later infected with virulent bacilli by the same route. The last named found no protection when the subsequent infection was given subcutaneously. Lange and Lydtin [9] found that the skin lesions caused by intradermal infection were more accelerated and less necrotic in animals vaccinated with B.C.G. by mouth than in control animals. King and Park [10] and Birkhaug [11] obtained no protection when peroral vaccination was followed by subcutaneous infection. Petroff and Steenken [12] had similar negative results with inhalation infection following peroral vaccination.

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The literature on parenteral vaccination is much more extensive. Among the best controlled and most extensive experiments on guinea pigs are those of Okell and Parish [13], Petroff and Steenken [12] and Birkhaug [11]. Okell and Parish found that a few of the vaccinated pigs were completely protected and that the mean survival times of the vaccinated animals were significantly greater than those of the controls. They obtained best results from intravenous vaccination followed by conjunctival infection with a very small dose of virulent bacilli, thus confirming the original experiments of Calmette, Boquet and Nègre [14]. Petroff and Steenken found the mean survival times of their vaccinated animals to be somewhat longer than those of the controls (differences of approximately twice their standard deviation according to our calculations from their figures). They obtained equal or greater protection in animals vaccinated with killed cultures of human tubercle bacilli, and definitely greater protection with a living attenuated human strain. Following the theory that allergy is detrimental to immunity and should be allowed to diminish, Birkhaug allowed longer intervals (17–19 weeks) to elapse between vaccination and infection. A few of his vaccinated animals survived and the mean survival times of his vaccinated groups were considerably greater than those of the controls. The magnitudes of the differences appear to be great enough to be definitely significant.

There is general agreement that the parenteral introduction of B.C.G. produces sensitivity to tuberculin in a large proportion of guinea pigs inoculated. There is less agreement as to the results of peroral vaccination. Petroff and Steenken [12] and King and Park [10] produced no sensitivity by feeding large doses of B.C.G. Lange and Lydtin [9], as previously mentioned, obtained accelerated lesions on the intradermal inoculation of virulent bacilli into guinea pigs vaccinated by mouth. Jensen, Moerch and Oerskov [15] were able to produce sensitivity to tuberculin in 73 per cent of new-born guinea pigs by feeding B.C.G. Birkhaug [11] in similar experiments produced sensitivity in 40 per cent of new-born guinea pigs.

Experimental:

The strain of B.C.G. and the methods of its culture and preparation have been described in the previous paper. The H 37 strain used for reinfection was kindly sent to us by Doctor Aronson of the Henry Phipps Institute, Philadelphia. It was cultured on glycerine-egg medium and transferred to glycerine-broth to obtain material for inoculation. The suspensions were prepared in the same manner as the B.C.G. suspensions.

Peroral vaccination of new-born guinea pigs:

Pregnant females were removed from the breeding cages to smaller cages containing 3 to 6 pregnant animals. These cages were inspected daily. When new-born pigs were present the mothers and young were removed.

The young in each litter were divided into two groups. One group was vaccinated; the other left as controls. One-half of the mothers and the vaccinated young were placed in a separate cage; the remainder of the mothers and the unvaccinated young in another cage. The supply of new-born pigs was limited and for each experiment the young were allowed to accumulate over a period of days. This resulted, within experiments, in some variation in the intervals between vaccination and infection. The feeding of the B.C.G was done by means of pipettes. Emulsions containing 100 mgm. per cc. were used. The emulsion was thoroughly shaken and then 1 cc. was taken in the pipette and 0.25 cc. amounts placed in the mouths of the young pigs which were held by an assistant. Only occasionally was any emulsion spilled or regurgitated. Daily feedings were given until the required amounts were administered. The animals suffered no apparent ill effects: the weight curves compared favorably with those of the controls and the death rates previous to infection were, in most cases, as small or smaller than those of the control groups. The vaccinated animals were kept separated from the control group during the vaccination and for one week afterwards. They were then all placed in the same cage. This usually coincided with the time of weaning when the mothers were returned to the breeding cages. The experiments were thus designed so that as far as possible, the only variable factor would be that of vaccination.

Parenteral vaccination:

Older guinea pigs of from 200 to 400 grams in weight were used for parenteral vaccination. In all cases an equal number of non-vaccinated control pigs of the same weight and age were placed in the same cage at the time of vaccination and later infected at the same time as the vaccinated pigs.

Tuberculin Tests:

All of the older animals used in the parenteral vaccination experiments were tested for tuberculin sensitivity prior to vaccination. In most (but not all) experiments all the animals were tested at intervals between vaccination and infection. The tests consisted of the intradermal injection of 0.1 cc. of a 1 in 20 dilution of Old Tuberculin (Mulford).

Reinfection:

Infection by mouth was done in the same manner as vaccination by mouth. The intradermal inoculations were made in the usual manner with tuberculin

syringes and 26 gauge needles. Emulsions were made so that the required doses would be contained in 0.25 cc. for oral administration and 0.1 cc. for intradermal inoculation. In both types of infection vaccinated and control pigs were infected alternately so as to eliminate inequalities which might arise from uneven suspensions or the settling of bacilli in the syringes. Daily observation and measurements were made of the lesions resulting from intradermal inoculation.

Evidences of Allergy following Vaccination.—All guinea pigs which were inoculated parenterally* with B.C.G. developed sensitivity to the amount of tuberculin used in 1 to 2 weeks. In those animals which were vaccinated intradermally and infected intradermally a marked acceleration of skin lesions occurred.

Our data on the development of allergy after peroral vaccination are incomplete, but the following observations were made: No sensitivity was produced in new-born guinea pigs by the dosages used (45, 75, and 150 mgm.) in less than 49 days; 150 mgm. produced sensitivity in 2 of 15 animals in this time. Following vaccination with 75 mgm., sensitivity appeared first in 57 days; at 66 to 71 days 5 of 11 animals tested reacted positively; from 75 to 94 days 11 of 18 animals were sensitive. Evidence of allergy in the acceleration of skin lesions produced by intradermal injection of H 37 was never obtained with 45 mgm. but the longest period allowed was 23 days. Nine of twelve animals infected intradermally from 75 to 85 days following vaccination with 150 mgm. showed accelerated skin lesions. Three adult guinea pigs weighing 400 grams, which were vaccinated with 150 mgm. of B.C.G. by mouth gave no tuberculin reaction in 29 days but 2 of 3 reacted positively in 35 days. This suggests that adult guinea pigs became allergic more rapidly than new-born pigs. In these experiments the duration of the sensitive state could not be determined since infection with H 37 was superimposed, but two new-born pigs vaccinated with 75 mgm. reacted in 132, and 177 days but failed to react 267 days following vaccination.

Measurement of protection against virulent infection afforded by vaccination with B.C.G.—As in all long continued

* These included a considerable number of animals which were not included in the tables and which were vaccinated subcutaneously and intraperitoneally.

experiments upon guinea pigs, intercurrent infection greatly increased the difficulties in the interpretation of results. These difficulties were further enhanced, in these experiments, by the occurrences of one minor and one major hurricane, both of which caused the deaths of many animals from exposure. The results of those experiments which could be completed in spite of these hindrances are summarized in the Tables I, II, III, and IV.

The factors stressed in interpreting the results were: (1) the mean survival times in each group; (2) the percentage of animals in each group with no macroscopic or microscopic tuberculosis. When animals were killed at the end of the experiment the interval from day of infection to day of killing was considered to be the survival time. Since most survivors were vaccinated animals, this tended to discriminate against the vaccination but was not frequent enough to make a very great difference. It seems likely that the end result of intercurrent infection would also be to conceal any possible beneficial results of vaccination by preventing the longer life of the vaccinated. The percentage of animals with no tuberculosis would seem to be the most important criterion in these experiments. This would introduce an error if a disproportionate number of animals in any one group tended to be killed by intercurrent infection very early after infection before the tuberculous disease had a chance to develop. This, however, was not the case. The animals tabulated as showing no tuberculosis or less than 4 + tuberculosis in many cases were the longest survivors and the others died at times when animals from the control groups were dying with extensive tuberculosis.

The percentage of animals with no tuberculosis and the mean survival times have been treated statistically to determine the importance which might be attached to the differences.*

Analysis of results—For calculating the standard deviations of the differences in the percentages of animals free from tuberculosis, the following formula was used:

* Professor E. B. Phelps of the Delamar Institute of Public Health, kindly suggested the methods to be used and supervised their application.

$$S.d. = 100\sqrt{p^\circ q^\circ\left(\frac{1}{n_v}+\frac{1}{n_c}\right)}$$

where: n_v = number of vaccinated animals

n_c = number of control animals

$q^\circ = \frac{\text{number of vaccinated free from tbc.} + \text{number of controls free}}{n_v + n_c}$

$p^\circ = 1 - q^\circ$

The standard deviations of the differences between the mean survival times were determined as follows:

$$S.d. = \sqrt{\sigma_{m_v}^2 + \sigma_{m_c}^2}$$

where: $\sigma_{m_v} = \sigma_T \frac{1}{\sqrt{n_v - 1}}$

$\sigma_{m_c} = \sigma_T \frac{1}{\sqrt{n_c - 1}}$

$\sigma_T = \sqrt{\frac{\epsilon\delta^2}{N}}$

N = Total number

n_v = Number of vaccinated

n_c = Number of controls

δ_x = Difference between mean days survival of total vaccinated animals and days survival of animal (x).

The survival times of all animals in each group were first charted as decimals of the means. The distribution was found to approximate a normal curve. Experiments sufficiently alike were combined to render statistical analysis more suitable.

Experiments 7 and 12: These experiments differed somewhat in that the interval between vaccination and infection was shorter and the infecting dose somewhat greater in *7* than in *12*. Likewise none of the animals in Experiment *7* showed any evidences of allergy previous to infection while more than 50 per cent of those in *12* reacted to tuberculin before infection. There was some evidence of protection in both groups but this was manifested in different ways. The difference between survival times was greater in Experiment *7*, but differences in amount of tuberculosis were present only in Experiment *12*. Since we have postulated the latter dif-

ferences to be of greater importance we interpret Experiment *12* as showing somewhat greater evidence of protection. In spite of these differences the experiments were considered to be sufficiently alike to combine for statistical analysis. The ratio of the difference of mean survival times to its standard deviation is 1.6. The odds against the result being due to chance are therefore 8 to 1. The ratio of the difference between percentages of animals free from tuberculous disease to its standard deviation is 1.5, making the odds against this difference being due to chance about 6½ to 1.

Experiments 14 and 15: The interval between vaccination and reinfection was shorter and the infective dose greater in Experiment *15* than in Experiment *14,* but the results in the two experiments are essentially alike. The ratio of the difference in mean survival times in the combined experiments to its standard deviation is 1.97. The odds against this difference being due to chance are 20 to 1. The ratio of the difference between the percentages of animals with no tuberculosis to its standard deviation is 2.65 making the odds against chance causation of this difference 106 to 1.

Experiments 3 and 4: The infecting dose was larger in Experiment *4* and this was reflected in a shorter average survival time in the controls of this group. In each of these experiments the mean survival time of the vaccinated group was less than that of the control group. This difference, in the combined experiments, was not as great as its standard deviation. The vaccination in these experiments had no demonstrable effect. (Table II.)

Experiments 6 and 7: The intervals between vaccination and infection were approximately the same. The amount of B.C.G. given was larger in Experiment *7.* The mean survival time was greater in the control group than in the vaccinated group. The ratio of the difference to its standard deviation is 1.4. The odds against chance causing this difference are 5 to 1. There were no differences in tuberculosis. There is a very slight indication that vaccination shortened the survival times of the vaccinated animals.

Experiments 9 and 10: The experiments were practically identical in set-up. The mean survival times were equal in

TABLE I

VACCINATION AND INFECTION BY MOUTH

Dose of B. C. G. Administered	Number of Animals at Start	Died before Infection	Interval between Vaccination and Infection	Infection Dose of H 37	Duration of Experiment	Number of Survivors at End of Experiment	Mean days of Survival	Number Free from Tuberculosis	Number with less than 4+ Tuberculosis*	Statistical Analysis, Combining Similar Experiments							
										Nature of Group	Number in Groups	Mean Survival Time			Freedom from Tuberculosis		
												Mean Days Survival	Difference of Means	Standard Deviation of Difference	Percent Free from Tuberculosis	Difference of Percent Free	Standard Deviation of Difference
75 mgm.	13	4	55–79 days	6×10 mgm.	322 days	1	231.5	0	0	Vacc.	27	219	37	22.25	7.4	7.4	4.9
0	13	4		"		0	177	0	0								
75 mgm.	24	6	88–13 days	2×10 mgm.	400	2	213.5	2	6	Controls	29	182			0		
0	23	3		"		0	184	0	2								
150 mgm.	21	6	55–70 days	5 mgm.	392	1	237	3	7	Vacc.	43	220	41	20.75	18.3	18.3	6.9
0	20	10		"		0	132	0	3								
150 mgm.	29	1	19–59 days	2×5 mgm.	393	4	212	5	7	Controls	35	179			0		
0	29	4		"		2	202	0	6								

TABLE II

VACCINATION BY MOUTH: INFECTION INTRADERMALLY

45 mgm.	6	1	23–26 days	1,200 bacilli	513 days	0	201	0	1	Vacc.	13	217	—33.9	44.5	7.6	—.7	
0	6	1		"		1	301	0	0								
75 mgm.	12	4	15–33 days	15,000 bacilli	547 days	1	228	1	1	Controls	12	245			8.3		
0	12	5		"		0	249	1	2								
75 mgm.	6	1	85 days	12,000 bacilli	305 days	0	138	0	1	Vacc.	17	180	—59	41	0		
0	6	4		"		1	241	0	0								
150 mgm.	17	5	85–98 days	8,000 bacilli	392 days	1	197	0	4	Controls	10	239			0		
"	17	9		"		3	238	0	4								

TABLE III

VACCINATION INTRADERMALLY: INFECTION INTRADERMALLY

[illegible]	[illegible]	2	70 days	10,000 bacilli	424 days	5	241	5	6	Vacc.	26	213	0		23	23	5.9
0	20	3		"		2	233	0	6								
[illegible]	[illegible]	0	68 days	7,000 bacilli	350 days	1	150	1	7	Controls	26	213					
						0	175	0	0								

vaccinated and control groups. The ratio of the difference between the percentages of animals with no tuberculosis to its standard deviation is 3.89. The odds against the result being due to chance are 10,000 to 1. In Experiment *10* all of the vaccinated animals had very little tuberculosis as compared with the controls. In spite of the lack of difference in survival times, which was probably due to intercurrent infection, the very definite difference in the extent of tuberculosis is good evidence of an immunizing effect of the vaccination. (Table III.)

The experiment does not include sufficient numbers to permit conclusions from slight differences. There is very little or no indication that vaccination had any effect. This experiment is not strictly comparable with those of Table III because the amount of B.C.G. given was less and the infective dose of H 37 (judging by the mean survival time of the controls) was greater. (Table IV.)

Discussion

The conditions of the experiments are too varied to justify conclusions regarding any other factor than that of vaccination itself. There is quite definite evidence that 150 mgm. of B.C.G. by mouth will give some protection against subsequent H 37 infection by mouth. Still more definite is the evidence that intradermal vaccination with B.C.G. will give some protection against intradermal infection with H 37. There is sufficient similarity between Experiments *6* and *13* on the one hand and *14* and *15* on the other to suggest that vaccination by mouth provides better protection against infection by mouth than it does against intradermal infection. Intradermal vaccination gives decidedly more protection against intradermal infection than does oral vaccination but it is impossible to determine from these experiments to what extent this difference might be due to an immunization of the portal of entry, or merely to the fact that peroral vaccination is generally less effective than intradermal. Experiment *11* suggests that intradermal vaccination is less effective in protecting against peroral infection than is peroral vaccination but the experiment is limited and not strictly comparable with those of Table I.

We have already referred to the indications in the literature that peroral vaccination provides better protection against peroral infection than it does against parenteral infection. Ornstein and Steinbach [16] found some evidence that intracutaneous immunization (with R 1) provided better protection against subsequent intracutaneous infection (H 305) than it did against subcutaneous infection.

Our data are not sufficiently extensive to draw conclusions regarding the relationship of allergy to protection. No relationship was evident in these experiments. The groups having the greatest degree of protection (Exps. *9, 10*) also contained the greatest percentage of animals sensitive to tuberculin before infection or showing accelerated skin lesions on intradermal reinfection. On the other hand Experiments *11* and *13* containing almost as great a percentage of allergic animals showed no evidence of protection.

Summary

An allergic state, manifested by the reaction to the intradermal injection of tuberculin or by an accelerated skin lesion following the intradermal injection of virulent tubercle bacilli, was produced regularly in guinea pigs by the parenteral introduction of B.C.G. Following the peroral administration of large doses of B.C.G. (75 and 150 mgm.) to new-born guinea pigs evidences of allergy were obtained in from 50 to 70 days; 75 per cent of the animals so treated were allergic after the latter period. The only two animals followed for sufficiently long periods remained sensitive for 177 days, but failed to react after 267 days. In a limited experiment, evidence was obtained that adult guinea pigs become allergic more rapidly following similar doses of B.C.G. by mouth than do new-born pigs.

The peroral vaccination of new-born guinea pigs with B.C.G. produced very slight but definite evidence of protection against peroral infection with H 37. Intradermal vaccination resulted in somewhat more marked protection against intradermal infection. There was some indication that vaccination by mouth gives better protection against peroral infection than it does against intradermal infection.

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