

DE SCHWEINITZ (E.A.)

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ITS USE IN PRODUCING IMMUNITY TO
TUBERCULOSIS IN GUINEA-PIGS.

BY

E. A. DE SCHWEINITZ, PH.D.,

BIOCHEMIC LABORATORY, BUREAU OF ANIMAL INDUSTRY, DEPARTMENT
OF AGRICULTURE, WASHINGTON, D. C.



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**THE ATTENUATED BACILLUS TUBERCULOSIS:
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BY E. A. DESCHWEINITZ, PH.D.,
BIOCHEMIC LABORATORY, BUREAU OF ANIMAL INDUSTRY, DEPARTMENT
OF AGRICULTURE, WASHINGTON, D.C.

APPARENT IMMUNITY TO TUBERCULOSIS IN
GUINEA-PIGS.

It is well known that a number of the pathogenic bacteria decrease very greatly in virulence when cultivated for a long time outside of the animal body. This fact has also been noticed in the case of the bacillus tuberculosis, and the literature is full of references to the decrease in virulence of the tubercle-bacillus and the way of securing this, but some facts have been developed in this connection in my work on tuberculin, in a new direction, which should be recorded.

Cultures with which I started several years ago were obtained through the kindness of Dr. Trudeau, of Saranac Lake, N. Y., and were the second generation on blood-serum from a rabbit. After growing for several generations upon glycerin-agar, about two months to each generation, the culture was transferred to glycerin beef-broth, which was to be used as a source for tuberculin. After the fourteenth generation I noticed that the guinea-pigs



that were always kept inoculated with tuberculosis, to test the tuberculin, did not succumb nearly as readily, and in the case of the fourteenth generation it required six months before the disease developed. I, consequently, inoculated a number of guinea-pigs with the seventeenth, eighteenth, nineteenth, and twentieth generations of this germ, which was alive and would grow very readily on the liquid media. Some of these pigs inoculated had previously been treated by an injection of tuberculin; the others had never been used. After some months those that had been inoculated with the attenuated germ remained quite well, and one was chloroformed, and the autopsy showed that it was free from disease.

In order to see if this might be accidental I then had nine pigs inoculated, four checks, four pigs previously inoculated with the attenuated germ, and one pig that had received tuberculin by feeding. These were all inoculated in the side with the material from the gland of a tuberculous cow that had just been killed. After seven weeks the checks were all found dead from tuberculosis. The other animals appeared perfectly well. One was accordingly chloroformed, and although very careful examinations were made no disease could be detected. Even the local lesion that was produced where the material had been injected had entirely healed. The animals were apparently immune.

Some experiments that had been made earlier with tuberculin prepared from a younger growth did not show that this material had a positive value in preventing disease, although it retarded it. As the preparation of tuberculin, however, continued,

I noticed that the tuberculin was much more constant in reaction and that a larger amount of the active principle was yielded by cultures of the older germ.

At this time I had at the Experiment-station of the Bureau the calf of a tuberculous cow, which, however, although the mother was badly diseased, did not respond to an injection with the tuberculin. The calf was then drenched with 100 c.c. of a growing active liquid culture of this attenuated germ, the twentieth generation. After three months the calf was again injected with tuberculin, and, failing to respond, was killed. The autopsy showed the animal to be perfectly healthy.

Another healthy Southern cow received into a vein an injection of the same generation from this liquid culture, in July. On November 20th this animal, which was rather thin and had an initial temperature of 103° F., was injected with tuberculin, but did not show any reaction. Another animal known to be tuberculous, injected at the same time, with the same-sized dose of tuberculin, showed a good reaction. The cow which had received the injection of attenuated culture had a small lump, the size of an egg, on the side of the neck, where inoculated. The diagnosis of no tuberculosis, as based upon failure of reaction to tuberculin, will be confirmed or disproved by an autopsy later. These experiments were kindly made for me by Dr. Schroeder.

So far as I have been able to find, the first note of successful cultivation of the tubercle-bacillus upon liquids free from albuminoid matter was made

by myself in the *New York Medical Journal* of March, 1893. Since then Uchinsky, Proskauer, and others have used similar media. None has, however, reported a better growth than that obtained by a slightly modified form of the original culture-medium. It required some time until I could persuade the germ to grow in this liquid, but after considerable coaxing it will now multiply as well here as on glycerin-peptonized beef-broth, the fourth generation having become accustomed to the change of food.

The formula that I have found eminently adapted and used may be repeated here:

Water	1000 cc.
Glycerin	70 grams
Acid pot. phos.	1 gram
Ammonium phos.	10 grams
Sodium chlorid	10 "
Asparagin	3 "
Magnesium Sulphate	0.2 gram

After a certain amount of growth has appeared in a definite volume of media the growth will entirely cease. The germ has exhausted the supply of nutritive material or eliminated enough poison to check its own development, and, although the germs may still float on the surface of the liquid, after from three to four months there is practically no growth in the liquid. Further, there is a distinct and peculiar odor generated in the culture-media. This is due to a fatty acid which is difficultly volatile with steam. The reaction of the media when inoculated is slightly alkaline. As the growth increases it becomes less so, and finally it is acid in reaction.

In order to see if the apparent loss of virulence of the germ might be due to some of its products contained in the body of the germ, two guinea-pigs were inoculated with the twenty-third generation of the germ, after the germs had been carefully and thoroughly washed with sterile water to remove all products. These pigs, after two months, are still well. The bacillus grown on purely artificial media might naturally be supposed to be still more attenuated than that grown on the ordinary culture-liquid. To test this, some of these germs were used to inoculate guinea-pigs, with the same results as those already noted.

In the case of a disease like tuberculosis, in which the incubation-period is often an exceedingly long one, there is a possibility that what now appear immune animals may in time succumb. In view of the fact, however, that all of the checks died so promptly, so long a time has elapsed since, and that all the other animals remained well, it is fair to presume that we have here a true immunity secured by an attenuated germ.

The following notes give the experiments in detail, and in the table they can be noted in a comprehensive form.

The autopsies are not given in full, as they appeared superfluous.

January 18, 1894. Guinea-pigs, Nos. 27, 28, 29, and 30, about three-quarters pound weight each, were inoculated with an emulsion of an agar-culture of tuberculosis, receiving 0.25 c.c. each, in the thigh. This was the eighteenth generation of the particular germ. On February 13, 1894, No. 27 was used to

test some tuberculin, and gave a good reaction, although apparently in good health. February 15, 1894, No. 28 was found dead, but the autopsy did not reveal any signs of tuberculosis. No. 27 was found dead on March 19th, two days after it had been fed a large amount of tuberculin. The autopsy did not reveal the presence of any tuberculous lesions. Nos. 29 and 30 were subsequently, within six months, chloroformed and examined, but no signs of tuberculosis were apparent.

February 16, 1894. Healthy guinea-pigs, Nos. 115, 113, 128, and 127, were fed varying doses of tuberculin, from 3 c.c. to 12 c.c. Nos. 127 and 128 succumbed to the feeding, but this proved to be due to the glycerin with which the tuberculin had been mixed.

On March 30, 1894, Nos. 114, 115, 113, 109, and 112 were inoculated with 0.1 c.c. of tuberculosis emulsion from the seventeenth generation of a liquid culture. June 5, 1894, No. 115 was chloroformed, but the autopsy did not reveal any signs of the disease. October 1, 1894, No. 114 was found dead outside of the cage; it had been killed by the rats and the head was badly eaten. The animal was in good condition and fat, and the autopsy did not reveal any signs of tuberculosis. There was a little thickening of the skin and a small non-tuberculous lump at the point of inoculation.

During April and May, Nos. 113 and 112 were fed on three different dates with small doses of tuberculin, but did not show any reaction. Both of these pigs are apparently in good health and entirely well at the present writing, eight months after inoculation. During this time guinea-pig No. 109 had been chloroformed, September 29, 1894, and examined without any signs of disease being apparent. The seventeenth generation of this germ was evidently too attenuated to cause death.

April 4, 1894. Guinea-pig No. 110 was fed with 9 c.c. tuberculin, diluted with sterile water without ill effect.

February 19, 1894, pig No. 122 was inoculated with 0.25 c.c. of tuberculosis culture, the seventeenth generation, and on April 4, 1894, was fed with 25 c.c. tuberculin. The pig was rather thin; there was a lesion the size of a pea at the point of inoculation, but the temperature was normal. April 9, 1894, this pig, No. 122, was found dead. The organs were apparently normal, except that the bloodvessels of the stomach and intestines were much congested. The pig had evidently been poisoned with the tuberculin; the contents of the intestines were thick and mucus-like.

February 19, 1894. Guinea-pig No. 121, weight 13 ounces, was inoculated with 0.25 c.c. tubercle culture. May 16th it was well and was reinoculated with 0.25 c.c. tubercle culture.

February 19, 1894. Guinea-pig No. 118 was inoculated with 0.25 c.c. of tubercle culture, the seventeenth generation. March 19th, this pig was found dead, but the autopsy did not reveal any signs of tuberculosis.

February 19, 1894. Guinea-pig No. 119, weight 14 ounces, received an injection of 0.2 c.c. tubercle culture, and on March 24, 1894, an injection of 0.5 c.c. of tuberculin. The temperature-reaction was very slight.

February 19, 1894. Guinea-pig No. 120, weight 14 ounces, was inoculated with 0.2 c.c. tubercle culture. On April 4, 1894, it was fed 3 c.c. of tuberculin. At this time it appeared to be ill, but did not show any reaction to the tuberculin, and the next day was quite well.

February 19, 1894. Guinea-pig No. 125 was inoculated with 0.25 c.c. tubercle culture. March

TABLE SHOWING EFFECT OF ATTENUATED TUBERCLE CULTURES AND

FEEDING TUBERCULIN IN GUINEA-PIGS, RABBIT, AND COW.

No. of animal.	Fed tuberculin.		Injected tuberculin.		Inoculated with artificial culture attenuated tubercle bacilli.	Fed tuberculin.		Injected tuberculin.		Inoculation with tub. gland.	Reinoculation with attenuated culture.	Dead.	Chloroformed.	Autopsy.		Present condition.
	Date.	Am't.	Date.	Am't.		Date.	Am't.	Date.	Am't.					Date.	Condition.	
Guinea-pigs	27	Jan. 18	Mar 17	24 c.c.	Feb. 13	1/4 c.c.	Mar 19	Mar. 19	Poisoned with tuberculin; no tuberculous.	Date of appearance, Nov. 28, 1894.
	28	Feb. 18	Well, fat, and in good condition.
	29	Feb. 18	Well, fat, and in good condition.
	30	Mar. 18	Well, fat, and in good condition.
	115	Feb. 16	6 c.c.	Mar. 30	June 5	June 5	Well, fat, and in good condition.
	113	April 5	4 c.c.	Well, fat, and in good condition.
	110	9 c.c.	Well, fat, and in good condition.
	141	Mar. 8	18 c.c.	May 16	Well, fat, and in good condition.
	142	26 c.c.	Well, fat, and in good condition.
	136	12 c.c.	Well, fat, and in good condition.
	135	Well, fat, and in good condition.
	137	Well, fat, and in good condition.
Rabbit	213	Well, fat, and in good condition.
	214	Checks	Well, fat, and in good condition.
	215	Well, fat, and in good condition.
	133	Well, fat, and in good condition.
	210	Well, fat, and in good condition.
	211	Well, fat, and in good condition.
	125	Well, fat, and in good condition.
	122	Well, fat, and in good condition.
	121	Well, fat, and in good condition.
	120	Well, fat, and in good condition.
	119	Well, fat, and in good condition.
	118	Well, fat, and in good condition.
Cow	114	Well, fat, and in good condition.
	109	Well, fat, and in good condition.
	112	Well, fat, and in good condition.
	141	Well, fat, and in good condition.
Cow	217	Well, fat, and in good condition.
	Well, fat, and in good condition.
	Well, fat, and in good condition.
	Well, fat, and in good condition.

Tested with tuberculin; no reaction November 20, 1894.

24th it received an injection of a small quantity of tuberculin, which caused a characteristic reaction.

March 8, 1894. Guinea-pig No. 141, three-quarters of a pound in weight, was given 18 c.c. of dilute tuberculin.

March 8, 1894. Guinea-pig No. 142, healthy, was fed 27 c.c. of tuberculin, but did not show any reaction. April 11, 1894, this pig was dead; the lungs were hemorrhagic, and the bloodvessels of the stomach and intestines much congested from tuberculin-poisoning.

March 12, 1894. Guinea-pig No. 136, healthy, was fed 12 c.c. of tuberculin.

May 16, 1894. Guinea-pig No. 135, weight one pound; guinea-pig No. 141, weight one pound; and guinea-pig No. 136, weight one pound, all received 0.25 c.c. tubercle culture, the twentieth generation. Guinea-pig No. 137, weight one pound, received 0.5 c.c. of the twentieth generation of tubercle culture. Nos. 141 and 136 had both been fed tuberculin once, the others had never been used.

July 27, 1894. Guinea-pigs Nos. 210 and 211, weight one pound each, received 0.5 c.c. of attenuated culture of tuberculosis, the twenty-second generation. At the present writing neither of these animals shows the least signs of tuberculosis.

July 29, 1894. Pigs Nos. 135, 136, 120, 110, 141, 125, and checks, healthy pigs, Nos. 213, 214, 215, and 133, were all inoculated for me at my request by Dr. V. A. Moore, with the material from the tuberculous gland of a badly-diseased cow, No. 286. The inoculations were made beneath the skin of the side. On September 11th checks Nos. 214 and 215 were both found dead, and the autopsy revealed extensive tuberculosis in lungs, liver, and spleen. September 21st, check No. 213 was found dead, the autopsy showing advanced tuberculosis. September

17th, guinea-pig No. 133 was chloroformed for examination, which revealed extensive tuberculosis.

October 13, 1894. Guinea-pig No. 136, one that had been inoculated in May with the attenuated culture, and subsequently inoculated with a tuberculous gland, was chloroformed for examination. Not a sign of disease could be detected and the local lesion from the inoculation had almost healed.

October 29, 1894. Guinea-pig No. 110 was found dead. This was a pig that had been fed upon tuberculin on April 4, 1894, and inoculated with the gland July 29, 1894. The autopsy showed advanced tuberculosis. The other pigs that had been inoculated with the attenuated culture and then the active virus, Nos. 135, 120, 141, and 125, are, at the present writing, quite well, and show no signs of disease. I may find that some of the animals that are now considered immune will eventually succumb to the disease. This, however, does not appear probable, as pig No. 136, examined after the death of all the checks, did not show any signs of disease.

Further, animals No. 137, inoculated with the twentieth generation May 16th, and No. 112, inoculated March 30th; Nos. 120 and 119, inoculated February 19th; No. 113, March 30th; and No. 121 on February 19th, and again on May 16th, are, at this writing, to all appearances perfectly well, having lived from six to nine months after the inoculation.

February 23d, rabbit No. 141 was inoculated with 0.25 c.c. of tubercle culture of the seventeenth generation. July 27th, this same rabbit was reinoculated with the twentieth generation of a tubercle culture, 0.25 c.c. being used. Still the animal is apparently well.

These experiments which, of course, in connection with so important and troublesome a disease, can be

regarded as preliminary only, and must be confirmed by a large number of similar ones, serve to show that by the use of attenuated tubercle-bacilli and their products we may be able to control pulmonary tuberculosis. If the twenty-second generation has shown such attenuation and produced so much resistance, then the thirtieth generation, or later, should give still more satisfactory results.

An examination of the table and accompanying notes will also show that tuberculin, when fed in very large doses to supposedly diseased or to healthy pigs, may cause the death of these animals by poisoning. The amount necessary varies from twelve to twenty times the quantity that would give a reaction in a tuberculous cow.

The digestion-experiments referred to in my report, in *Bulletin No. 7, Bureau of Animal Industry, Department of Agriculture*, have shown that the action of tuberculin upon diseased animals is not affected after twenty-four hours in a hydrochloric-acid pepsin-solution, nor by warming with acetic acid. On the other hand, in two cases noted, feeding the tuberculin in small doses, while it did not prevent the disease, apparently retarded it. Possibly a combined feeding of tuberculin and the inoculation with the attenuated germ may be found more useful than either alone.

The fact must not be lost sight of that the number of bacilli injected into an animal may cause great differences in the length of time of the development of the disease. There could be no error of this sort, however, in the experiments recorded, as the emulsion of virulent and attenuated cultures

used for inoculation were prepared in the same way, and a larger quantity of the attenuated culture was used than is necessary in case of a more virulent one to produce death, in five to six weeks.

What conclusions may be drawn from these experiments? We have guinea-pigs made apparently distinctly immune to an active tuberculous virus by the use of an attenuated germ. We have guinea-pigs in which a single injection of a small quantity of tuberculin increased, perhaps, the subsequent immunity secured by injection with the attenuated culture. We have other pigs the immunity of which has not been tested, it is true, by an active virus, but which were inoculated with the attenuated culture many months ago, and are still well, and in which an autopsy of other animals treated in exactly the same way and at the same time showed no signs of disease.

Recently Viquerat claims to have been able by means of subcutaneous injections of the serum of the ass, an animal naturally immune to tuberculosis, to effect cures and improvement in the disease in man. Our attenuated germ may possibly prove very valuable in checking or controlling tuberculosis in animals, especially cattle.

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