

THE SURVIVAL OF THE CALMETTE-GUÉRIN STRAIN OF TUBERCLE BACILLUS (B.C.G.) IN THE BLOOD AND MAMMA OF GOATS

BY THE LATE A. STANLEY GRIFFITH, C.B.E., M.D., PH.D.
Formerly Member of the Scientific Staff, Medical Research Council

From the Field Laboratories, Cambridge

ONE of the objections to the use of B.C.G. in cattle as an immunizing agent against infection with tuberculosis was the possibility of these micro-organisms establishing themselves in the mammary sinuses and being excreted with the milk. There would, of course, be no disadvantage in this if the bacilli in these surroundings did not regain the virulence they were supposed at one time to have possessed. It may be recalled that the originators of B.C.G. claimed that the strain had descended from bovine bacilli which had been cultivated from a tuberculous cow and had lost their virulence during artificial cultivation. My observations, many as yet unpublished, agree with the opinion expressed by Calmette that the pathogenic properties, such as they are, of B.C.G. are fixed and cannot be enhanced by passage through animals or by growth in or on artificial media (deep broth cultures, etc.). If this view of the stability of the characters of B.C.G. be correct, the appearance of this micro-organism in the milk of cows would not constitute a danger to the health of human beings and might even prove an advantage, since such milk might have an immunizing effect against infection with tuberculosis on those who drink it.

In the past, living cultures of human tubercle bacilli have been used in heifer calves as vaccines in ignorance of the fact that the bacilli may pass into the sinuses of the immature udder, flourish therein until such time as the heifer has a calf, and be expelled with the milk when lactation begins (Griffith, 1913, 1917).

It has also been shown that attenuated bovine strains of low virulence for the calf may be excreted into the sinuses of the immature udder without causing tuberculosis of that organ (Griffith, 1911). But no instance, to my knowledge, of the colonization with B.C.G. of the udder of a vaccinated heifer has been observed. It is not indeed known whether or not B.C.G. injected by way of the teat canal directly into the mammary sinuses would become established therein and what effect their presence there would have upon the mammary gland itself. Virulent bovine bacilli injected in this way generally caused an acute widespread tuberculosis of the infected quarters which was rapidly fatal from toxæmia. On the other hand, human bacilli of normal virulence rarely produced any structural change in the udder beyond fibrosis, but persisted in the milk sinuses and tubules for very long periods during which

they could be demonstrated in the milk by injection of guinea-pigs (Cobbett, 1917). Injected intravenously into cows human bacilli are excreted with the milk and persist in the sinuses. In one instance they gave rise to caseo-calcareous nodules in the udder (Griffith, 1911).

Goats which have been injected parenterally with living mammalian tubercle bacilli excrete these bacilli with the milk, as do cows. When working for the Royal Commission on Tuberculosis I (Griffith, 1911) injected five goats with human or with bovine tubercle bacilli and found these bacilli in the milk in three instances 24 hr., in one instance 48 hr. and in one instance 14 days after injection; the bacilli persisted in the udder in each case. Avian tubercle bacilli may also pass into the milk of goats after their inoculation subcutaneously. A notable example of their excretion into the udder of a goat and persistence therein for a period of about 9 years may be referred to (Griffith, 1938).

As a contribution to the inquiry into the fate of B.C.G. in the mamma I injected two goats through the teat canals with this micro-organism and subsequently made cultures with the products milked from the infected udders.

Goat 165. This goat had been previously used in 1934 in an experiment with B.C.G. This experiment was made to ascertain (1) how long B.C.G. circulates in the blood after primary and subsequent intravenous injections, and (2) whether or not B.C.G. is excreted into the mammary sinuses after injection into the blood. The results are now reported for the first time.

On 28 May 1934 the goat, a nanny about 2 years of age, was injected intravenously with B.C.G. and received subsequently three further spaced intravenous injections of B.C.G., the dose on each occasion being 10.0 mg. of culture. After each injection blood, usually 10.0 c.c., was withdrawn from the opposite jugular vein and citrated, and cultures were made from it according to the method of Loewenstein. The results of these tests are summarized in Table 1.

The centrifuged deposits were sown on plain egg and glycerin egg, each tube receiving an amount of the centrifuged deposit which was representative of 1.0–1.25 c.c. of blood. The results were sometimes very irregular, some tubes in a batch growing a number of colonies, others none at all, though each tube was treated in precisely the same way.

In three tests the citrated blood was used for sowing the tubes directly. After the third injection one drop of the 24 hr. blood produced three B.C.G. colonies. After the fourth injection the 1 min. and the 24 hr. blood samples were cultivated directly; of the 1 min. sample two amounts each of 0.025 c.c. yielded 50 and 36 colonies respectively, while 0.05 c.c. yielded 15 colonies only; of the 24 hr. sample 0.075 c.c. produced one colony only on the three egg tubes sown. These results show that a larger proportion of the tubercle bacilli can be cultivated from the blood by the direct method than by Loewenstein's method, provided that the amount of blood used is not more than is necessary to spread thinly over the surface of the egg, namely, about 0.025 c.c.

Table 1

Date	Intervals at which blood collected and numbers of colonies				
	1-1½ min.	24 hr.	48 hr.	72 hr.	Later
20. ii. 34	67 cols. on 7 tubes. 3 tubes sterile	8 cols. on 6 tubes. 2 sterile*	2 cols. on 2 tubes. 5 sterile	1 col. on 1 tube. 7 sterile	1 col. from 8th day blood, 7 tubes sterile. On 6th, 10th and 14th days 23 tubes in all sterile
28. v. 34	41 cols. on 3 tubes. 2 contam., 1 sterile	—	1 col. only, 4 tubes sterile, 1 contam.	6 tubes, 5 sterile, 1 contam.	No B.C.G. cols. on 4th, 7th and 9th days
16. x. 34†	61 cols. on 3 tubes (each c.d. 1 c.c. blood)	72 cols on 3 egg tubes; 3 cols. on 3 glyc. egg tubes; 2 egg tubes sterile (each c.d. 1-25 c.c. blood)	14 cols. on 6 tubes from c.d. 12 c.c. blood. 3 tubes sterile	—	2 cols. on 12 tubes, c.d. of 10 c.c. blood on 4th day. Blood on 7th day sterile
18. iii. 35‡	136 cols. on 4 tubes. 2 contam.	35 cols. on 6 tubes (32 cols. on 2 and 3 cols. on 3 tubes, 1 tube sterile)	18 cols. from c.d. 8 c.c. blood	1 col. from 6 c.c. blood	No B.C.G. from 5th and 7th days' blood

c.d. = centrifuged deposit.

* 4-0 c.c. blood inoculated into a guinea-pig which was killed 30 days later. P.M.—No tuberculosis and cultures from inguinal glands negative.

† Febrile reaction after this injection. Temp. next day 41.5° C. and high (40.2° C.) until seventh day.

‡ Injection followed by moderate febrile reaction, highest temperature 39.6° C.

After each injection viable B.C.G. was abundant in the blood withdrawn from the opposite jugular vein 1-1½ min. later and quickly diminished. After the first injection the eighth day blood again grew one colony, the sixth day blood having proved negative. It is probable that B.C.G. was not circulating in the blood up to the eighth day, but was released on that day from some spot where the micro-organism had been held up.

These results may be compared with those obtained with the blood of a goat (No. 159) injected by me in connexion with the international inquiry of the Health Committee of the League of Nations into Loewenstein's technique of blood culture (Griffith, 1933). Goat 159 was injected intravenously in the left jugular vein with 50.0 mg. of B.C.G. The goat died of pneumonia 9 days after injection, the lungs being in a state of red hepatization without visible tubercles. Blood was withdrawn from the right jugular vein 1, 5 and 24 hr. and 2, 3, 5, 6 and 9 days after the goat was injected. B.C.G. was recovered from all the specimens, the 1 hr. blood producing numerous colonies and the others colonies in progressively diminishing numbers. From the last specimen one colony only of B.C.G. was obtained. The longer persistence of the micro-organism in the blood of this goat than in that of goat 165 is attributable to the larger dose of B.C.G. given to the former.

It may be mentioned that Loewenstein examined in Vienna duplicate samples of the blood specimens and sent to me two virulent cultures of human type which were stated to have been grown from the 3 and the 5 days' specimens, duplicate specimens of which at Cambridge had yielded colonies of B.C.G. only.

In January 1935 the mamma was immature (the goat had not had a kid) and perfectly dry.

Each mammary cistern was washed out with 10.0 c.c. sterile physiological salt solution, and 7 c.c. of slightly milky fluid were recovered from one and 9 c.c. from the other half of the mamma. These amounts were centrifuged, the deposits being used to sow egg tubes. The tubes remained sterile.

Intramammary injection. On 16 April 1935 the goat received intramammary injections of B.C.G. through the teat canals. Two different strains were used, both of which had been sent to me by Prof. A. Calmette, one in 1922 and one in 1927 (246 and 467 generations respectively on bile potato). The dose for each side was 20.0 mg. emulsified in salt solution, the first strain on the right, the second on the left side. A febrile reaction followed the injection, the temperature rising to 41.4° C. at 10 a.m. and 40.9° C. at 4 p.m. on the next day and falling to 40.5° C. on the morning of the second day. Blood drawn from the jugular vein 24 hr. after the goat was injected in the mamma proved sterile. Ten egg tubes were sown, two each with one drop of citrate blood and eight with the centrifuged deposit of 4.0 c.c. blood. It was thought that the B.C.G. intramammarily injected, after producing a preliminary local reaction, would in due course either all be eliminated or become permanently established in the mamma as was the case with the avian bacillus in goat 98 (Griffith, 1938). The injections were, however, followed by pronounced inflammatory reactions, the milk cisterns and teat canals becoming filled with sero- and caseo-pus, some of which was removed periodically by milking to relieve tension and for examination. The sero-pus came away easily, but the caseo-pus, except the thinner portions, could not at first be expressed.

For some time the cisterns were filled with caseo-pus and the mammary gland itself was enlarged, indurated and nodular. Gradually the caseo-pus liquefied and was evacuated, but the udder did not become dry until 16 months after injection.

Microscopical examinations of the pus were made from time to time; acid-fast bacilli were easily found on every occasion, but were less numerous at the end than at first. The early preparations showed degenerate epithelium cells and numerous polymorphonuclear leucocytes, many of which were stuffed with acid-fast bacilli which were also lying free. Later the leucocytes disintegrated and clumps of acid-fast bacilli were seen surrounding broken-down nuclei. Later still the pus showed acid-fast bacilli only and no cellular elements.

Cultures were also sown and B.C.G. was cultivated from the pus 11, 51, 78 and 101 days after injection. The viable bacilli were numerous at first and soon diminished, the sowings on the last occasion yielding only one colony from one

half and none from the other half of the mamma. All subsequent sowings, the last on 11 May 1936, were sterile.

A kid was born on 29 May 1936, but though it sucked it was unable to obtain sufficient nourishment and died on the second day.

Another kid was born on 30 April 1937, and because there was no mammary secretion was killed. The autopsy showed no sign of disease. The goat was tested intradermally on 22 October 1935 with mammalian tuberculin by Mr R. E. Glover, F.R.C.V.S.; a definite reaction was obtained. The nanny-goat was killed on 6 May 1937, 2 years and 20 days after the intramammary injection.

Autopsy. The mammary gland was much enlarged and of an elastic firmness without definite nodules; it measured $6\frac{1}{2}$ in. in length, 5 in. across and $2\frac{1}{2}$ in. in depth. Nothing could be milked from the teat, the canal of which was not patent. On section through the mamma the tissue was pinkish, very dense and fibroid and showed small milk cysts. A very little milk oozed from the cut surface which showed scattered granules of calcareous substance. A scraping of cut surface showed scanty acid-fast bacilli. The cisterns were almost obliterated, the surfaces of the remaining walls showing some foci of calcareo-caseous substance containing acid-fast bacilli. Internally there was no sign of tuberculosis. The lung parenchyma seemed a little firmer than normal.

Goat 167, virgin nanny, 2-3 years old, was injected up the teat canal of the right mamma on 15 January 1935, with a suspension of culture of B.C.G. The mamma was very small and there was insufficient space for 9 c.c. of suspension, some of which escaped. The dose was estimated to be in the neighbourhood of 40.0 mg.

Clinical history. Next day the right teat canal was distended with fluid. On the fourth day the right mamma measured 4 in. across at the base and 3 in. in depth and was very hard. After 13 February the mamma began to diminish a little in size and became knotty posteriorly and the mammary lymphatic gland was obviously enlarged. On 29 April for the first time a small quantity of thick caseo-pus was expressed from the teat and subsequently at irregular intervals about 0.5 c.c. or less of similar substance was squeezed out. For some time the mamma measured about $4 \times 3 \times 2\frac{1}{2}$ in., the lower part being fluctuant, the upper hard and knotty. The abscess in the milk cistern broke through the skin in front of the teat after 18 September 1937.

Film preparations of the pus always showed acid-fast bacilli. The first pus seemed composed of polymorphonuclear leucocytes, rather degenerate, many of which contained acid-fast bacilli which were moderately numerous. The acid-fast bacilli diminished in number in the course of the experiment, but were always easily found. Cultures were sown from the first pus obtained, namely, 104 days after the experiment began; the tubes remained sterile. At intervals subsequently specimens of pus, the last taken from the mamma at autopsy, were tested by culture. No B.C.G. colonies were obtained; the micro-organisms which were seen on microscopical examination were dead. The goat reacted to

tuberculin 91 days after inoculation. The tuberculin was produced by the growth of a eugonic human strain on whey of cows milk, 0.1 c.c. of the undiluted fluid being injected intradermally. The animal reacted again on 22 October 1935, and on 18 September 1937, ordinary mammalian tuberculin being used. The goat was kept with a billy but did not become pregnant.

The goat was killed 3 years and 9 days after inoculation.

Autopsy. Right half of mamma measured $4 \times 3 \times 1$ in: In front of the teat there was a small depressed opening which communicated with the mammary cistern, on the walls of which some whitish pus was present. The rest of the mamma was composed of white cheesy nodules 2 mm. up to 1.5 cm. in diameter, gritty from calcification, each surrounded by a thin wall of fibrous tissue. These were embedded in a scanty connective tissue matrix. The right teat canal was obliterated. The left mamma was atrophied and showed nothing abnormal. The right supramammary lymphatic gland was larger than the left (hyperplastic) but otherwise unaffected. There was no sign of tuberculosis internally. As previously stated, acid-fast bacilli were easily found in the nodular and cistern pus, but were not viable.

SUMMARY

Intravenously injected into a goat in a single dose of 50.0 mg. B.C.G. caused the death of the animal from pneumonia in 9 days and was demonstrated in the blood until the day of death, though in progressively diminishing numbers.

Intravenously injected into a goat on successive occasions, the dose on each being 10.0 mg., B.C.G. was filtered from the blood by the tissues within 3–5 days. There was little or no evidence that B.C.G. disappeared from the blood more quickly after previous injection of that micro-organism. B.C.G. was not excreted into the milk cisterns of this goat or, if excreted, the bacilli did not long survive.

Intramammarily injected B.C.G. produced inflammatory reactions in the mamma with formation of caseo-pus and ultimate destruction of the gland as a secretory organ. The micro-organisms, however, did not remain alive for any lengthy period in the mamma and in the two instances reported were not viable after about 15 weeks.

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