STUDIES ON B. C. G. *

I. THE PATHOGENICITY OF B.C.G.

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The majority of workers who have investigated the pathogenicity of B.C.G. for laboratory animals agree that it produces tuberculous lesions at the site of inoculation, with involvement of the regional lymph nodes and, occasionally, isolated tubercles of the spleen, liver or lungs. They maintain, however, that these lesions are always benign and heal without the development of any progressive disease. A not inconsiderable minority have reported that, in some instances, progressive, fatal tuberculosis has been produced by B.C.G. No attempt will be made, in this paper, to review the enormous literature on B.C.G. Reference will be made only to those authors whose work has direct bearing on the experiments reported here. More or less adequate summaries of the literature may be found in a series of reviews [1, 2, 3, 4, 5, 6 and 7] and in recent papers by Birkhaug [8] and Behner [9].

Petroff and his co-workers [10, 11] found that B.C.G. grown on gentian-violet egg medium produced two types of colonies, an "R" (resistant to environment) type and an "S" (sensitive) type. On Sauton's medium the "R" grew in isolated islands, while the "S" type produced a thin, spreading veil. They found that the "S" type invariably produced progressive tuberculosis in guinea pigs and occasionally in rabbits. Begbie [12] and Reed [13] confirmed the occurrence of a variant, pathogenic, colonial type. Sasano and Medlar [14] in 1931 reported that they had made B.C.G. virulent for rabbits, guinea pigs and calves by growing it for a number of passages on Sauton's medium to which 10 per cent normal rabbit serum had been added. The growth on this medium became veil-like, similar to the "S" form of Petroff. Other work-

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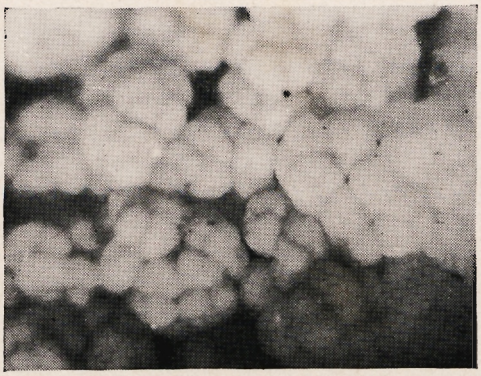
ers, while confirming the existence of different colonial forms, have not found that any form produced progressive disease. Neufeld [15] reported that Petroff's "S" cultures were of the human type of bacillus, suggesting that they were contaminants. Many other workers, including most recently, Christison [16], Birkhaug [8] and Behner [9], could not produce pathogenic variants by Petroff's methods. Boquet [17] and Behner [9] were unable to confirm the observations of Sasano and Medlar [14].

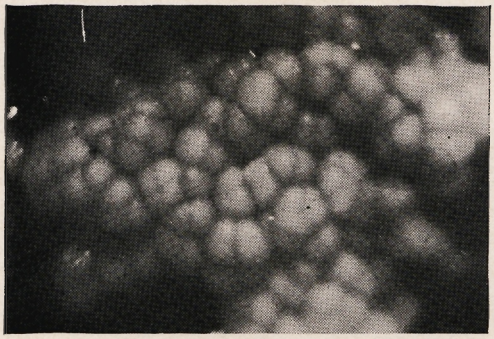
A different method of producing pathogenic variants was used by Dreyer and Vollum [18] who grew B.C.G. in the bottoms of large flasks of bouillon instead of on the surfaces. They produced generalized tuberculosis in guinea pigs with the second and fourth generations of such cultures. Meissner and Prausnitz [19], Birkhaug [8], Behner [9] and others were unable to repeat these experiments.

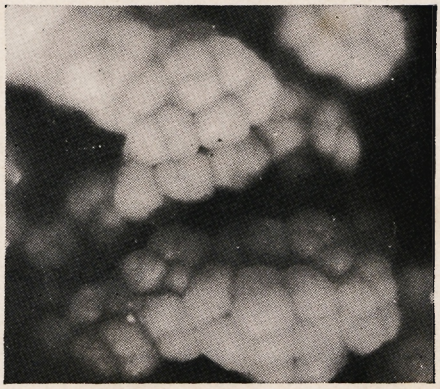
Characteristics of "Island" and "Veil" types of growth. —Close observations of B.C.G.* cultures grown on Sauton's medium showed that they usually consisted of more or less isolated clusters of small uniform units very much resembling clusters of grapes (see illustration). Occasionally, in certain flasks, a thin veil-like growth, microscopically revealing no definite structure, would extend out of the typical island masses and rapidly cover the whole surface of the medium. When a piece of this "veil" was transferred to a new flask an apparently pure culture of "veil" was produced. However, this "veil" growth never remained as such, either when allowed to continue growth in the same flask or when transferred. The "island" type always reappeared and eventually replaced the "veil" type. The latter would reappear on subsequent transfers and the cycle would be repeated. The reversion of "veil" to "island" was more regular and certain than the reverse.

Numerous measurements of the hydrogen-ion concentration of cultures of apparently pure "veil" and "island" growths were made at various times after inoculation up to 40 days. The measurements were made colorimetrically, using fresh Lamotte indicators and standards. The pH was

* The strain of B.C.G. used in these experiments was obtained from Professor Calmette in September 1931, labelled 41FS$_2$27/8. Except where otherwise stated it was carried on bile-potato alternating with glycerine-potato, and transferred to Sauton's medium to obtain growth for inoculation.







B.C.G. "Island" growth on Sauton's medium. 25 days growth. Approximately 25×.

never below 6.8 with either type of growth. Both gave identical curves typical for bovine organisms. Control measurements of H 37 cultures by the same methods showed typical human-type curves with the pH as low as 5.00 on the 25th to 40th days. Petroff[11] reported that the "S" veil type of B.C.G. produced a pH of 5.5 to 6.4 and differed in this respect from the "R" strains.

Animal inoculation with "island" and "veil" growths:

Preparation of suspensions: growths from 21-to-28-day-old cultures on Sauton's medium were squeezed between sterile absorbent paper to remove as much moisture as possible. After being weighed on an analytical balance the cultures were emulsified with saline by long-continued grinding in a mortar. Saline was added to make the required number of milligrams per cubic centimeter. When direct bacterial counts were to be made the emulsion was centrifuged at moderate speed to remove all clumps. The centrifugation was controlled by examination of stained smears of the slightly cloudy supernatant to detect clumps of more than 3 or 4 bacilli. Areas measuring 1 by 2 cm. were marked by diamond pencil on specially cleaned slides. A thin film of normal rabbit serum was allowed to dry on these marked rectangles to serve as fixative. By means of a 0.1 cc. pipette graduated to thousandths of a cc., five thousandths of a cc. of the supernatant was spread over the marked rectangle as evenly as possible and allowed to dry. The slides were stained with Ziehl-Niehlsen stain in the usual way. Three slides were prepared from each emulsion. The visual field of the microscope was delimited by an adjustable square mask in the eye piece which was arranged so that exactly .01 square millimeter was included in the field. By using the mechanical stage 50 fields evenly distributed over the 2 square centimeters were counted on each slide. The results of the three slides were averaged. The emulsion was then diluted to give the required number of bacilli per cc.

Table I shows the details and results of the injection of 20 guinea pigs, by different routes, employing various doses of the two types of B.C.G. growth.

No tuberculous lesions were found macroscopically or microscopically in any of these animals. In animals inoculated intradermally, swollen, reddened, indurated lesions appeared at the sites of the inoculation within 2 or 3 days. Sometimes these lesions became ulcerated but always regressed and eventually disappeared.

Inoculation of B.C.G. grown on Sauton's medium containing 10 per cent rabbit serum.—Sauton's medium containing 3 per cent glycerine was adjusted to pH 7.3 and 10 per cent fresh normal rabbit serum added according to the method of Sasano and Medlar[14]. The growth of B.C.G. on this medium was more rapid and more profuse than that on the

TABLE I*

INOCULATION OF GUINEA PIGS WITH "VEIL" AND "ISLAND" TYPES OF GROWTH

Type of Growth	Number of Guinea Pigs	Route of Injection	Weight or Number of B. C. G.	Results
Veil	2	Intradermal	1,000.000	One died 40 days...
				No tuberculosis.
				One killed 300 days.
				No tuberculosis.
	3	Intradermal	3 mgm 6 mgm.	All killed 520 days..
				No tuberculosis.
	3	Subcutaneous	4,000,000	All killed 300 days.
				No tuberculosis.
	2	Intraperitoneal	12 mgm.	Both killed 520 days.
			25 mgm.	(1 Pseudotuberculosis°)
Island	2	Intradermal	1,000,000.	Both killed 300 days.
				No tuberculosis.
	2	Intradermal	3 mgm 6 mgm	Both killed 520 days.
				No tuberculosis.
	3	Subcutaneous	4,000,000.	All killed 300 days
				(1 Pseudotuberculosis °)
	4	Intraperitoneal	12 mgm.	One died 420 days.
			25 mgm.	Two killed 260 days.
				One killed 520 days.
				No tuberculosis.

*In this and all later experiments all guinea pigs were examined grossly for evidences of tuberculosis in the lymph glands, spleen, lungs and liver. These organs were also sectioned and examined microscopically.

°See section on "Pseudotuberculosis".

TABLE II

INOCULATION OF GUINEA PIGS WITH B. C. G. GROWN ON 10% SERUM IN SAUTON'S MEDIUM

Generation	Number of Guinea Pigs Inoculated	Amount of B .C. G.	Route of Inoculation	Results
Second	2	10 mgm.	Intraperitoneal	1 died 60 days.
				No tuberculosis
				1 killed 260 days.
				No tuberculosis.
Third	13	10-100 mgm.	Id.	2 died in 30 and 37 days.
				Local tuberculous lesions of peritoreum and omentum.
				1 died 97 days.
				No tuberculosis (Pseudotuberculosis*)
				2 died 320 and 360 days.
				No tuberculosis.
				1 killed 245 days.
				No tuberculosis.
				7 killed 450 days.
				No tuberculosis.

*See section on pseudotuberculosis.

usual Sauton's medium inoculated at the same time. The "veil" type of growth appeared early, and rapidly predominated. Only small outgrowths of "veil" appeared in the control flasks. Three consecutive transfers were made on the serum and allowed to grow respectively 26, 38 and 15 days. Guinea pigs were inoculated with the second and third generation cultures. Table II gives the details and results of these inoculations.

Two rabbits were injected intravenously with 10 mgm. of the second generation culture and one rabbit was given 10 mgm. of the third generation culture. The first two, killed in 260 days, showed no evidence of tuberculosis. The third rabbit died twelve days after the injection. The lungs were filled with coalescent tubercles which contained acid-fast bacilli. The kidneys contained 2 or 3 very small tubercles.

TABLE No. III

INOCULATION OF GUINEA PIGS AND RABBITS WITH B. C. G. GROWN IN DEEP BROTH CULTURE

	Number of Animals	Amount of Culture	Route of Injection	Results
Guinea Pigs	5	2 to 20 mgm	Intraperitoneal.....	All killed 250 days. No tuberculosis.
Rabbits....	2	25 to 30 mgm...	Intravenous...	Killed 25 days. No tuberculosis
	1	5 mgm	Intraperitonal.....	Killed 25 days. No tuberculosis.
	1	10 mgm.....	Into artery....	Killed 25 days. No tuberculosis.

Inoculation of animals with B.C.G. grown in deep broth. —Flasks of 500 cc. of broth as used by Dreyer and Vollum [18] were prepared and inoculated with large amounts of growth from a 25-day Sauton's culture of B.C.G. The inoculum was caused to sink to the bottom of the flasks. After 22 days' incubation there appeared to be an increase in the amount of granular sediment. Transfers of this sediment to a new series of such flasks were made by the means of pipettes. After 24 days' incubation there was a definite increase in the quantity of sediment, and low power magnifications showed shaggy outgrowths from the granular masses. Table III gives the details and results of animal inoculations made with this material from the second series of flasks.

PSEUDOTUBERCULOSIS

A condition designated "pseudotuberculosis" was found in a number of guinea pigs during these experiments on pathogenicity and during those on protection reported in the following paper. It was characterized, grossly, by the presence of small yellowish white spots about 1 mm. in diameter on the spleen, liver and lungs. These organs were often a deep hemorrhagic red in color. Microscopically the lesions consisted of foci of hemorrhage and necrosis infiltrated by fragmented leukocytes and surrounded by large mononuclear phagocytes which at times resembled epithelioid cells. Dense clumps of rather large pleomorphic non-acidfast bacilli were found in these areas.

The condition was found in 28 of 259 guinea pigs which received various doses of B.C.G. by different routes and was found only 3 times in 216 guinea pigs kept in similar conditions and often in the same cages, which were infected only with H.37. It occurred in 5 of 50 animals which received only B.C.G. The incidence was irregular, being greater in some cages than in others. Since equal numbers of vaccinated and control pigs were kept in the same cages the difference in incidence between the two groups was not merely due to cage differences. The great discrepancy in the incidence of this condition was not appreciated until the experiments were concluded, so that further investigations of it were not made.

Since, in most cases, the guinea pigs involved had little or no true tuberculous disease, it seems likely that the difference in incidence was due to an inhibitory or masking effect of the true tuberculosis when this was more extensive. The alternative explanation, that B.C.G. made the animals more susceptible, does not seem likely in view of the failure of B.C.G. to increase the death rates prior to reinfection (see the following paper).

Discussion

The results of these experiments are in agreement with those of the majority of workers who have investigated the potential pathogenicity of B.C.G. A local tuberculous process is produced at the site of inoculation, but this lesion recedes and does not give rise to generalized disease. The tubercles of the lungs and kidneys produced by the intravenous injection of 10 mgm. of the third generation of serum culture might possibly be interpreted as an indication of pathogenicity, but it is likely that 10 mgm. of killed culture injected intravenously would sometimes produce the same picture.

Aside from the differences in interpretation as to what constitutes progressive tuberculosis, there are essentially two possible reasons for the disagreement regarding the pathogenicity of B.C.G.: (1) Those workers reporting the production of progressive tuberculosis have been working with contaminated cultures or have met with spontaneous tuberculosis in their experimental animals. The implication regarding the human type qualities of Petroff's "S" organisms

have already been mentioned. Calmette repeatedly stressed that spontaneous tuberculosis of guinea pigs is much more frequent than generally supposed. (2) B.C.G. strains vary in their pathogenicity. Behner has pointed out that positive reports of virulence are much less frequent in recent than in former years, and suggests that the longer culture on bile-potato has further decreased its potential pathogenicity. It is true that several of the workers claiming pathogenicity for B.C.G. have experienced marked variations in the pathogenicity of different strains (Petroff, Watson, Dreyer and Vollum). Watson found a graded decrease in virulence in the 1924, 1925 and 1927 strains.

Summary

B.C.G. strain 41FS$_2$27/8 grew on Sauton's medium in the form of "veil" and "island" growths. Both forms gave pH curves typical of bovine organisms. Neither "veil" nor "island" growths produced progressive tuberculosis in guinea pigs. This strain of B.C.G. cultured on 10 per cent fresh rabbit serum according to the method of Sasano and Medlar, or in deep broth according to the method of Dreyer and Vollum, produced no evidence of progressive tuberculosis when injected into guinea pigs or rabbits.

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