VARIABILITY OF BCG STRAINS (BACILLUS CALMETTE-GUÉRIN)

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Plates 47 to 49

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The results of recent studies suggest that there may exist some correlation between the morphological growth pattern of mammalian tubercle bacilli and their virulence for experimental animals (1, 2). In cultures of all virulent strains, the organisms have a tendency to adhere one to the other in the direction of their long axis, thus forming serpentine strands (so called "cords") in liquid media, and spreading colonies on the surface of agar media. By contrast three cultures tested *in vivo*, because they had been shown to grow without any orientation (H37Ra, JH16Ra, R1Ra), have proven to be completely devoid of virulence. These avirulent cultures form amorphous clumps in liquid media and non-spreading colonies on agar (3).

Although it is easy to differentiate morphologically the fully virulent strains from the avirulent Ra variants, the morphology of the attenuated[1] strain BCG is not so readily defined (1). This is probably due to the fact,—which appears from the data to be presented in the following pages,—that the various cultures of BCG in use throughout the world are heterogeneous with reference to their colonial morphology. It has been found, on the other hand, that these cultures also differ in their ability to cause lesions in experimental animals. Accordingly, it has appeared worthwhile to investigate in greater detail the relation between the pattern of growth of BCG cultures and their behavior *in vivo*.

EXPERIMENTAL

Four different cultures of BCG were received in our laboratory on the following dates:

BCG I—received in 1947 from Dr. J. D. Aronson of The Henry Phipps Institute, Philadelphia. This culture had been sent to Philadelphia by Dr. Bretey of the Pasteur Institute of Paris on April 4, 1946.

BCG II—received on May 15, 1947, from Dr. S. R. Rosenthal of the Tice Laboratory, Chicago. This culture was labeled BCG 839 K.

BCG III—received on May 21, 1947, from Dr. J. D. Aronson of The Henry Phipps Institute, Philadelphia. This culture was labeled BCG 793 (series 2).

[1] For reasons given here and elsewhere (4), it seems best not to refer to BCG as "avirulent" but to qualify it instead as "attenuated."

BCG IV—received on December 13, 1949, from Dr. K. Birkhaug of the New York State Department of Health, Albany. This culture was labeled BCG 805 (series 2).

The media used have been described earlier in detail (5, 6). The cultures were transferred weekly or biweekly in liquid medium containing 0.02 per cent Tween 80 and 0.5 per cent albumin and were incubated at 37°C. for 10 days. They were then stored at 4°C. in the ice box until used. Under these conditions the cultures are known to remain viable for many weeks (7).

In order to obtain preparations suitable for the study of colonial morphology, the cultures were diluted 1000- or 10,000-fold in autoclaved distilled water containing 0.1 per cent albumin, and a volume of 0.02 or 0.05 ml. of these dilutions was deposited and spread on the surface of agar media containing oleic acid, albumin, and 0.25 per cent glucose (6). The agar plates were sealed with adhesive tape or later kept in plastic bags as described by Powell (8). As the shape of the colony is influenced by moisture, it was found important to maintain a fairly constant degree of humidity throughout the period of incubation.

After 14 to 21 days of incubation at 37°C., the colonies were examined with a binocular dissecting microscope. They were classified as belonging to the spreading, intermediate, or non-spreading type, according to criteria to be defined in another section of this paper. The data on distribution of the three colonial types are based upon counts of two hundred colonies per plate. The figures are presented as percentages.

An attempt was also made to determine the pattern of growth in terms of the tightness of the "cords" formed in the depth of liquid or semisolid agar media (basal medium with oleic acid and albumin). The semisolid media contained 0.1 per cent agar, and quantities of 2.0 ml. of it were distributed into centrifuge tubes. The inoculum was 0.2 ml. of culture diluted 1000-fold; the tubes were sealed with cotton plugs soaked with paraffin and were incubated at 37°C. Microcolonies appeared after 5 to 7 days, and 2.0 ml. of sterile water was then added to each tube. The tubes were placed in a boiling water bath for 10 minutes, and their contents were centrifuged for 10 minutes at 2000 R.P.M. The supernatant fluid was removed, and the sediment spread on a glass slide was stained by the Ziehl-Neelsen technique. The tightness of the cords was recorded according to an arbitrary scale from 0 to 6.

Colonial Forms Observed in the Parent BCG Cultures.—Three types of colonies have been distinguished. The colonies of the *spreading* type have a dense and opaque center surrounded by a halo which consists of serpentine strands folded close together (Fig. 5). Differences in the tightness of the cords are made evident by comparing a spreading colony in Fig. 5 with one in Fig. 2. In the *intermediate colonial* type the dark center is much larger, and the halo is reduced to a small band. The serpentine pattern can still be seen (Fig. 1, top, and Fig. 3, bottom). Opacity and lack of orientation are characteristic of the *non-spreading colony*. A rudimentary halo is sometimes observed (Figs. 3 and 6).

The relative proportions of these three colonial types and the character of the cords in the parent BCG cultures are indicated in Table I. Colonies of the intermediate type predominate in strains I and II, which produce only a few colonies of the spreading type (Fig. 1). The cords are moderately tight in strain I and very loose in strain II. Characteristic for strain III are the high proportion of spreading colonies and the formation of tight cords (Fig. 4). The same is true of strain IV. The cultures of BCG can, therefore, be distinguished by two morphological criteria—the relative proportion of the three colonial forms and

the appearance of the cords. As will be shown in the following experiment, these properties remained characteristic of each culture during cultivation *in vitro.*

Isolation of Substrains and Their Morphological Characteristics.—

Spreading and non-spreading colonies were isolated from plates inoculated with the parent ultures of BCG, and they were grown in the liquid Tween-albumin medium. The subcultures were then diluted and inoculated on the surface of solid media, and the resulting growth was examined to determine the distribution of spreading or non-spreading colonies. The substrains were subcultured in the liquid medium for further study.

TABLE I

Relative Proportions of Colonial Types and Intensity of Cord Formation in Three Strains of BCG

Strain	Distribution of colonial types*			Intensity of cord formation‡
	Spreading	Intermediate	Non-spreading	
	per cent	*per cent*	*per cent*	
I	5	85	10	1–3
II	5	75	20	0–2
III	80	15	5	3–5

* Colonies grown on the surface of agar media.

‡ Microcolonies grown in the depth of semisolid media. The degree of cord formation is expressed according to an arbitrary scale from 0 to 6, 6 corresponding to the morphology of fully virulent strains.

The substrains propagated from isolated colonies should be readily differentiated from the parent cultures. As shown in Table II those derived from spreading colonies consisted almost exclusively of the spreading colonial type.

The spreading substrains isolated from strain III—the most spreading of the parent cultures—proved to be particularly homogeneous and showed the tightest cords (Fig. 5). On the other hand, the spreading substrains of I and II were less homogeneous, always containing a certain proportion of intermediate and non-spreading colonies (Fig. 2). Cord formation was less pronounced in substrain II than in substrain I. The substrains derived from non-spreading colonies were a mixture of intermediate and non-spreading colonies. The substrains obtained from BCG I and II yielded similar numbers of both intermediate and non-spreading colonies, with a few of the spreading type (Fig. 3). The substrain derived from BCG III was more uniform with a great majority of non-spreading colonies (Fig. 6).

Table III illustrates the fact that completely homogeneous substrains could not be obtained, even after repeated isolation of apparently pure non-spreading colonies. As expected, the non-spreading substrains failed to form cords, or did so only to a very limited degree.

Stability of the Substrains during Cultivation in Vitro and Animal Passage.— The findings that have just been reported reveal that BCG cultures are made up

TABLE II

Relative Proportions of Colonial Types and Intensity of Cord Formation in Spreading and Non-Spreading Substrains Derived from Three BCG Strains

Substrains*	Distribution of colonial types‡			Intensity of cord formation§
	Spreading	Intermediate	Non-spreading	
	per cent	*per cent*	*per cent*	
I a	90	10	0	4–5
b	2	60	38	0–1
II a	90	8	2	2–3
b	3	60	37	0–1
III a	100	0	0	5–6
b	1	24	75	1

* a = spreading substrain; b = non-spreading substrain.

‡ Colonies grown on the surface of agar media.

§ Microcolonies grown in the depth of semisolid media. The degree of cord formation is expressed according to an arbitrary scale from 0 to 6, 6 corresponding to the morphology of fully virulent strains.

TABLE III

Relative Proportions of Colonial Types in the Non-Spreading Substrain of BCG Strain II after Repeated Isolation of Non-Spreading Colonies

Isolation	Transfers*	Distribution of colonial types‡		
		Spreading	Intermediate	Non-spreading
		per cent	*per cent*	*per cent*
1	4	1	40	59
	8	1	49	50
2	1	5	55	40
	2	3	52	45
3	1	1	49	50
	2	2	60	38

* Number of transfers in liquid media after each isolation.

‡ Colonies grown on the surface of agar media.

of a morphologically heterogeneous population and that the relative proportions of the colonial types can be changed to favor the prevalence of either the spreading or the non-spreading colonial type. It is a remarkable fact that, notwithstanding this potential variability of their morphological characteristics, the cultures of BCG remain fairly constant, not only in the course of

repeated transfers *in vitro* but also during passages in mice and guinea pigs. This is illustrated in the following experiment.

White mice of the Rockefeller Institute strain were injected either intravenously or intracerebrally with 0.1 or 0.03 ml., respectively, of undiluted culture grown in liquid medium containing Tween and albumin. The mice were killed with chloroform from 1 day to 5 weeks after infection. Tissues were removed aseptically and ground in a mortar with sterile alundum sand. Dilutions of the suspensions were prepared with sterile distilled water containing 0.1 per cent bovine albumin. Agar plates and, in some cases, tubes containing liquid medium were inoculated with these dilutions. The subcultures obtained in liquid media were also inoculated

TABLE IV

Relative Proportions of Colonial Types in Spreading and Non-Spreading Substrains of BCG Strains II and III through Several in Vitro Passages

Strain	Distribution of colonial types*							
	Spreading substrain				Non-spreading substrain			
	n	Spreading	Intermediate	Non-spreading	n	Spreading	Intermediate	Non-spreading
		per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*
II	2	95	5	0	4	1	40	59
	5	84	16	0	6	0	40	60
	8	95	5	0	10	0	50	50
	9	96	4	0	14	1	70	29
III	1	100	0	0	1	0	25	75
	2	100	0	0	2	0	20	80
	3	100	0	0	3	0	36	64
	4	100	0	0	4	0	13	87

* Colonies grown on the surface of agar media.
n = number of transfers in liquid medium containing Tween 80 and albumin.

on the surface of agar media. The morphology of the resulting growth was recorded as described in the preceding experiments.

Guinea pigs were infected by the intraperitoneal route with undiluted cultures. They were killed with chloroform 4 or 12 weeks later, and suspensions of their tissues were then inoculated on agar media. Typical examples of the morphological composition of the strains recovered after repeated *in vitro* transfers and after passage through animals are given in Tables IV and V.

The cultures recovered from mice or guinea pigs consisted, in general, of the same proportion of the three colonial types that was found in the cultures used for infection. The morphological composition of cultures of BCG I and its substrains before intravenous injection into mice and after recovery from the spleens of infected animals is recorded in Table V. As can be seen, the morphological characteristics of the strains were identical before and after passage through mice. Moreover, the results were independent of the time elapsed between infection and recovery, being the same after 1 day or 5 weeks.

It is apparent, therefore, that no selective effect on any of the colonial types could be obtained either by passage through animals or by subcultivation *in vitro*.

Similar experiments were performed with culture IV. As in the case of BCG I, II, and III, this strain was found to consist of a mixed colonial population from which spreading and non-spreading substrains could be separated. This is a fact of some interest since, in contrast to the other cultures that had been maintained in our laboratory for long periods of time, culture BCG IV had been obtained directly from a culture used for routine preparation of the vaccine.

The Degree of Attenuation of the Various Strains of BCG and of the Substrains Derived from Them.—The evaluation of the residual virulence of attenuated strains is not easy. By common usage, virulent strains are capable of producing

TABLE V

*Relative Proportions of Colonial Types in BCG Strain I and Its Spreading and Non-Spreading Substrains before and after Passage through Mice**

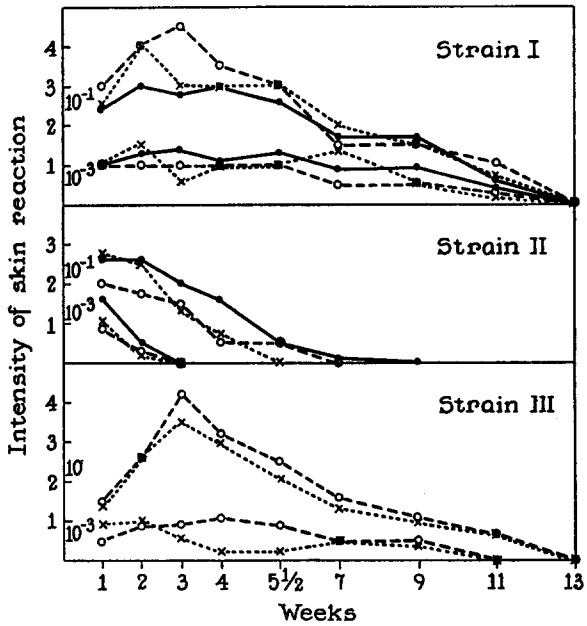
Strain	Distribution of colonial types					
	Before mouse passage			After mouse passage		
	Spreading	Intermediate	Non-spreading	Spreading	Intermediate	Non-spreading
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Parent	5	78	17	4	93	3
Spreading	88	12	0	85	15	0
Non-spreading	0	40	60	1	38	61

* The mice were infected intravenously with 0.1 ml. of undiluted culture and killed 5 weeks later. No macroscopic lesions could be found in the lungs. The strains were recovered from the spleens in liquid medium and dilutions of these cultures inoculated on agar media.

progressive and fatal disease, whereas avirulent strains are unable to proliferate *in vivo*. Thus, mortality of infected experimental animals is a sufficient criterion to differentiate between these two groups of strains. But it cannot be used to compare the behavior *in vivo* of attenuated strains since these produce only a self-limited disease. In this case, the degree of residual virulence (attenuation) must be judged from the extent and nature of the lesions found at a given time after infection and from the time required for tissue repair to take place. Judgment is rendered more difficult by the fact that, for a given strain, these two data are affected by the number of viable bacilli administered. The degree of attenuation of the BCG cultures studied in the present report was measured in the light of these considerations.

It has been shown by others that in guinea pigs infected intracutaneously with graded amounts of tubercle bacilli, the intensity and duration of the skin lesions can serve as an index of the degree of attenuation of the strains injected (9, 10). This was confirmed in the following experiment.

Albino guinea pigs of the Rockefeller Institute strain, weighing 350 to 450 gm., were used. The BCG cultures were grown for 8 days in the basal medium containing 0.02 per cent Tween 80 and 0.5 per cent albumin. The optical density of the cultures was measured in the Coleman junior spectrophotometer at a wave length of 550 mμ. Whenever necessary, the cultures were adjusted to the same optical density either by dilution or by concentration. Bacteriological counts were made according to a technique described elsewhere (11). The undiluted cultures contained between 30 and 50 million living units per ml. 0.1 ml. of culture either undiluted



TEXT-FIG. 1. Intensity of the skin reactions in guinea pigs infected intracutaneously with dilutions of three different strains of BCG and their substrains. By reactions is meant lesions produced by injection of cultures diluted 10- or 1000-fold. Each curve corresponds to an average of readings made on three to five different guinea pigs. The intensity of the reaction is recorded on the ordinate according to an arbitrary scale ranging from 1 to 4, 3 and 4 indicating the formation of an ulcer. ●———● parent strain, O- - - - -O spreading substrain, X- - - - -X non-spreading substrain.

or diluted 10-, 100-, 1000-, and 10,000-fold was injected intradermally on the back of the guinea pigs. Each animal received one or two strains, and each strain was injected into three to five animals. Readings of the intensity of the skin reactions were made weekly and later, biweekly. The lesions obtained with 10- and 100-fold dilutions of the cultures are recorded in Text-fig. 1.

In a second experiment, larger doses of cultures were injected intraperitoneally into guinea pigs in the hope of bringing out between the substrains differences that would not be detected otherwise (12). Colored male guinea pigs obtained from the Carworth Farms and weighing 400 to 500 gm. were infected with 5.0 ml. of 7-day-old cultures in Tween-albumin medium. Five animals were used for each bacterial strain. According to the bacteriological

counts made on agar plates, the suspensions contained 150 to 180 million living units per ml. Two animals of each group were killed with chloroform 4 weeks after infection and the remaining animals, 12 weeks later. The extent of involvement of the omenta, of the regional mesenteric lymph nodes, and of the spleens and the lungs was recorded. From tissues fixed in 4 per cent formaldehyde in physiological saline sections were made and stained with hematoxylin-eosin. As the alterations presented by the different animals within each group were

TABLE VI

*Effects of Intraperitoneal Injection of Large Amounts of BCG into Guinea Pigs**

Strain	Killed after	Macroscopic findings						Microscopic findings		
		Peritoneum	Omentum‡§		Regional lymph node‡	Spleen‡	Lungs‖	Regional lymph node¶	Spleen¶	Lungs**
	wks.									
I										
Spreading	4	Adhesions	++++	+	+	−	++	Not done	++++	++++
	12	Adhesions	++++	+	+	−	++	Not done	Not done	Not done
II										
Spreading	4	Adhesions	+++	+	−	−	−	Not done	+	+
	12	Normal	++	+	−	±	−	+	++	+/++
Non-spreading	4	Adhesions	++	+	−	−	−	Not done	+	+
	12	Adhesions	+	−	−	+	−	Not done	++	+/++
III										
Spreading	4	Adhesions	++++	+	+	++	+	++	+++	++/+++
	12	Adhesions	++++	+	+	+	+	Not done	++/+++	++
Non-spreading	4	Adhesions	++++	+	+	+	+	+/++	++/+++	++/+++
	12	Adhesions	+++	+	+	−	+	++	++/+++	+++

*Macroscopic and microscopic findings in guinea pigs having received intraperitoneally 5 ml. of undiluted culture of the spreading substrain of BCG I, or of the spreading or non-spreading substrains of BCG II and III. The findings recorded refer to two animals of each group; one killed 4, and the other, 12 weeks after infection.

‡Enlargement.

§+ = caseated, − = non-caseated.

‖+ = very few and small lesions, ++ = numerous lesions.

¶Enlargement of the organ and intensity of epithelioid infiltration.

**Number and size of foci.

found to be fairly uniform, only the findings in two animals of each group are recorded in Table VI—one killed 4 weeks, the other, 12 weeks after infection.

The results presented in Text-fig. 1 and Table VI agree in showing that strain I caused the most intensive and strain II, the weakest reactions, strain III being intermediate between the two but more like I than II. It is of special interest, furthermore, that the spreading and non-spreading substrains seemed to elicit tissue reactions almost identical with those of their respective parent strains and could not be differentiated by these criteria.

The skin lesions produced in guinea pigs at the site of injection of cultures of strains I and III (diluted 10-fold) ulcerated within 3 weeks after injection and were healed 13 weeks later. Differences between these two strains could be brought out by injecting cultures diluted 1000-fold. The lesions caused by strain II, after the 1st week, were of almost the same intensity as those caused by the other two strains. The former healed more rapidly—within 8 to 9 weeks—whereas the lesions due to the latter were ulcerating.

Differences of the same order were observed among the three strains in the second experiment. All animals killed either 4 or 12 weeks after infection had adhesions of the peritoneal layers, especially between the liver and omentum and between the omentum and spleen. The omenta were enlarged and contained caseated masses which were more firm at the 12 week period. The mesenteric lymph nodes were swollen in the guinea pigs infected with strains I and III, but normal in those having received strain II. Only strain III caused enlargement of the spleen, whereas the largest macroscopically visible lesions in the lung were found in animals infected with culture I. The lesions elicited by culture III were smaller in size and in number. No lesions were found in animals injected with culture II.

The differences in the lesions caused by the three strains appeared more clearly when tissue sections were studied microscopically. The character of the lesions was the same in all cases, the foci consisting of an accumulation of epithelioid cells well demarcated from the surrounding tissue. But the number and size of the lesions in the lung and the intensity of the cellular reaction in the regional lymph nodes and spleens were characteristic for each strain. The few lung lesions produced by culture II were small, and only slight changes were found in lymph nodes and spleens. More and larger lesions were present in lungs of animals infected with strain III, and small and large foci of epithelioid cells were found in the lymph nodes and spleens. More severe changes were caused by culture I. The lung lesions produced by the spreading substrains of the three BCG cultures studied are illustrated in Figs. 7 to 9.

Worth emphasizing again is the fact that microscopic studies, like macroscopic observations, failed to reveal any obvious difference in the lesions caused by spreading and non-spreading variants of each BCG strain.

Virulence tests were also carried out in mice, using strains of animals known to exhibit high susceptibility to tuberculous infection (13). Evaluation of the comparative pathogenicity of the different bacterial cultures was based on the extent of the pathological process observed in animals killed at certain intervals after infection.

The strains of mice C57 black, CF_1, and dba were used. The animals were obtained from Carworth Farms and kept for at least 1 week in our animal room before infection. Cultures grown for 7 to 9 days in the Tween-albumin liquid medium were injected by the intravenous route in 0.1 or 0.2 ml. amounts. The bacterial suspensions were adjusted to the same optical

density, and bacteriological counts were made to determine the number of living units per volume. The three parent bacterial cultures were compared with the spreading and non-spreading substrains derived from them. Ten mice were injected with each strain and either kept in groups of five in glass jars or of ten in metal cages. The animals were fed daily bread and milk. Half of them were killed with chloroform 5 weeks after infection, and the second half after 7 or 12 weeks. The number and character of the lung lesions and the size of the spleen were recorded. In some cases, the lungs were fixed in 4 per cent formaldehyde for microscopic study. A summary of the results of one experiment is given in Table VII.

TABLE VII

Comparative Virulence for Mice of BCG Strains I, II, and III and of Substrains Derived from Them

Strain	Extent of lesions		
	Macroscopic		Microscopic
	Lungs	Spleen	Lungs
I			
Parent	+++++	+	Not done
Spreading	+++++	+	Not done
Non-spreading	++++/+++++	+	Not done
II			
Parent	0	+	Not done
Spreading	0	+	+/++
Non-spreading	0	+	++
III			
Spreading	++/+++	+/++	+++++
Non-spreading	++	+/++	++++/+++++

0.1 ml. of undiluted culture containing 80 to 100 million living units per ml. was injected intravenously into C57 black mice. The extent of the pulmonary lesions and the size of the spleens of animals killed 5 weeks after infection are recorded in terms of an arbitrary scale ranging from + to +++++.

The only evidence of infection in mice injected with strain II was a slightly enlarged spleen. By contrast, many lung lesions were found in mice injected with the parent strains, as well as with the spreading and non-spreading substrains, of BCG I and III. The lesions were usually larger, of greater number, and more confluent in mice injected with strain I, whereas the lesions produced by strain III were small and isolated.

Microscopic inspection of sections of the lungs confirmed the difference of pathogenicity between BCG II and III (see Table VII). The lesions differed in number and size. Strain II caused very small foci consisting of a few cells, whereas the lesions were large and confluent in the lungs of mice injected with the substrains of BCG III. There were seen in those foci large cells with a

foamy protoplasm not found in lesions produced by strain II. The lungs of some of the animals injected with the spreading substrain of BCG III showed a greater number of lesions than the lungs of the animals injected with the non-spreading variant.

DISCUSSION

Although BCG is usually regarded as having reached a stable level of attenuation (12), there have been several reports that its behavior *in vitro* and *in vivo* can undergo modifications. The changes that have been reported are of multiple nature. (*a*) Increase in pathogenicity attributed to the occurrence of variants that exhibit a characteristic colonial morphology and that are fully virulent (14). (*b*) Alteration of colonial morphology not accompanied by detectable changes of virulence (15, 16). (*c*) Slight increase or decrease of virulence occurring during *in vitro* cultivation and revealed by animal experiments or by observations in man (10); some authors regard changes of this sort merely as artifacts due to differences in the "vitality" of the culture caused by inadequate growth conditions (17–19). (*d*) Modification of antigenic properties resulting from prolonged cultivation in the various laboratories engaged in preparation of the vaccine (20–22).

It is difficult to correlate the many claims and reconcile the conflicting views that have been published with reference to the variability of BCG. This is due in part to the fact that the published data are based on tests performed under a great variety of experimental conditions, and that quantitative criteria are still lacking for an adequate comparison of the bacterial preparations used by the different observers. It is meaningless, for example, to describe in terms of weight or volume the amount of culture injected, in the absence of knowledge of the percentage of cells that are viable and of their physiological condition. Unfortunately, no technique is available at present for an accurate description of these essential data.

In the present study an attempt has been made to minimize the difficulties involved in the comparison of cultures. This has been done by using identical conditions for the growth of the different cultures of BCG under study, and by infecting animals with bacterial suspensions of the same age containing the same number of living cells (within the limits in which these numbers can be determined) (10).

When infection experiments were performed with control of all known variables, it was found that three cultures of BCG obtained from as many laboratories differed widely in their ability to produce pathological changes. Two of the cultures, BCG I and III, caused macroscopic lesions in mice and guinea pigs, the lesions produced by the former being the more severe. The third culture (BCG II) did not induce any macroscopic lesions in the tissues of guinea pigs or mice, except the lesions produced in the skin of guinea pigs at

the site of injection. Since the cultures had been cultivated under identical conditions for several years in our laboratory, it appears inevitable to attribute their differences in pathogenic properties to intrinsic characteristics independent of environmental factors.

The BCG cultures under study were found to consist of morphologically heterogeneous populations as revealed by study of the colonial morphology and of the microscopic pattern of growth. Each strain produces on the surface of agar media a characteristic proportion of spreading, intermediate, and non-spreading colonies, and this proportion remained fairly stable during cultivation *in vitro* and passage in experimental animals. The least pathogenic culture (BCG II) produced a great number of intermediate and non-spreading colonies and proved to be a poor cord former, whereas cultures of BCG III were characterized by a high proportion of spreading colonies with maximal cord formation. Strain I was intermediate between the two, although it was the most pathogenic.

From the three BCG cultures there could be isolated substrains producing higher proportions of either spreading or non-spreading colonies. But despite their striking differences in ability to form cords, the spreading and non-spreading substrains were found to exhibit the same pathogenic potency as the parent cultures from which they were derived. It seems, therefore, that the ability of the BCG cultures to multiply or to survive in the tissues of infected animals is conditioned by factors independent of those manifested in the tendency to form cords or spreading colonies. This statement gains further support from the findings recorded in Table V, where it is shown that cultures of the non-spreading colonial type can be recovered as frequently as those of the spreading type from tissues of animals infected with one or the other, respectively. Thus, the *in vivo* environment does not appear to exhibit any selective inhibitory or destructive effect on the non-spreading forms.

The results that have just been discussed strongly suggest that attenuation of virulence of BCG cultures represents a type of variation different in nature from that corresponding to the Rv → Ra variation in which the loss of pathogenicity is accompanied by a change of morphological properties that expresses itself in failure to grow in the form of cords and of spreading colonies (1, 2, 3). Facts that may have a bearing on the mechanism of this new type of variation will be reported from this laboratory in forthcoming publications.

SUMMARY

Three different cultures of BCG propagated for over 2 years in a liquid medium containing Tween 80 and albumin were found to differ in several of their intrinsic properties.

Cultures of the three strains were found to consist of morphologically heterogeneous populations—each culture being made up of three main colonial

types—spreading, intermediate, and non-spreading. The percentage distribution of colonial types was characteristic for each culture and remained constant during cultivation in liquid media.

Injection of the various cultures into mice and guinea pigs resulted in a self-limited disease. The distribution, extent, and duration of the lesions were also characteristic for each culture.

Both the spreading and non-spreading substrains derived from the various cultures exhibited the degree of attenuation of virulence characteristic of the parent strain.

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EXPLANATION OF PLATES

The photographs were made by Mr. Julian Carlile.

PLATE 47

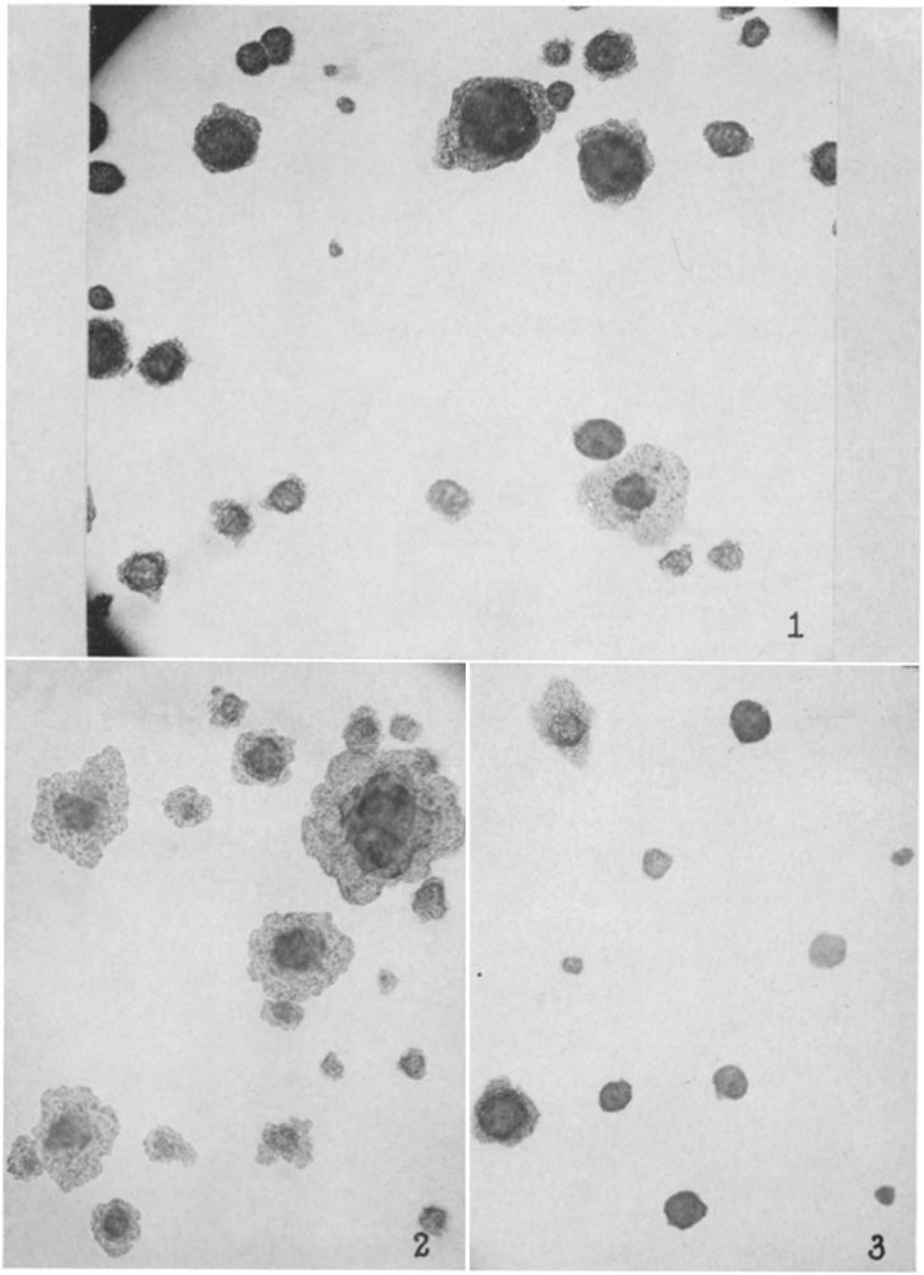
Colonial morphology of strain BCG II and of its substrains.

FIG. 1. Parent strain.

FIG. 2. Spreading substrain.

FIG. 3. Non-spreading substrain.

All colonies grown for 14 days on agar media containing oleic acid and bovine albumin. × 73.



(Suter and Dubos: Variability of BCG strains)

PLATE 48

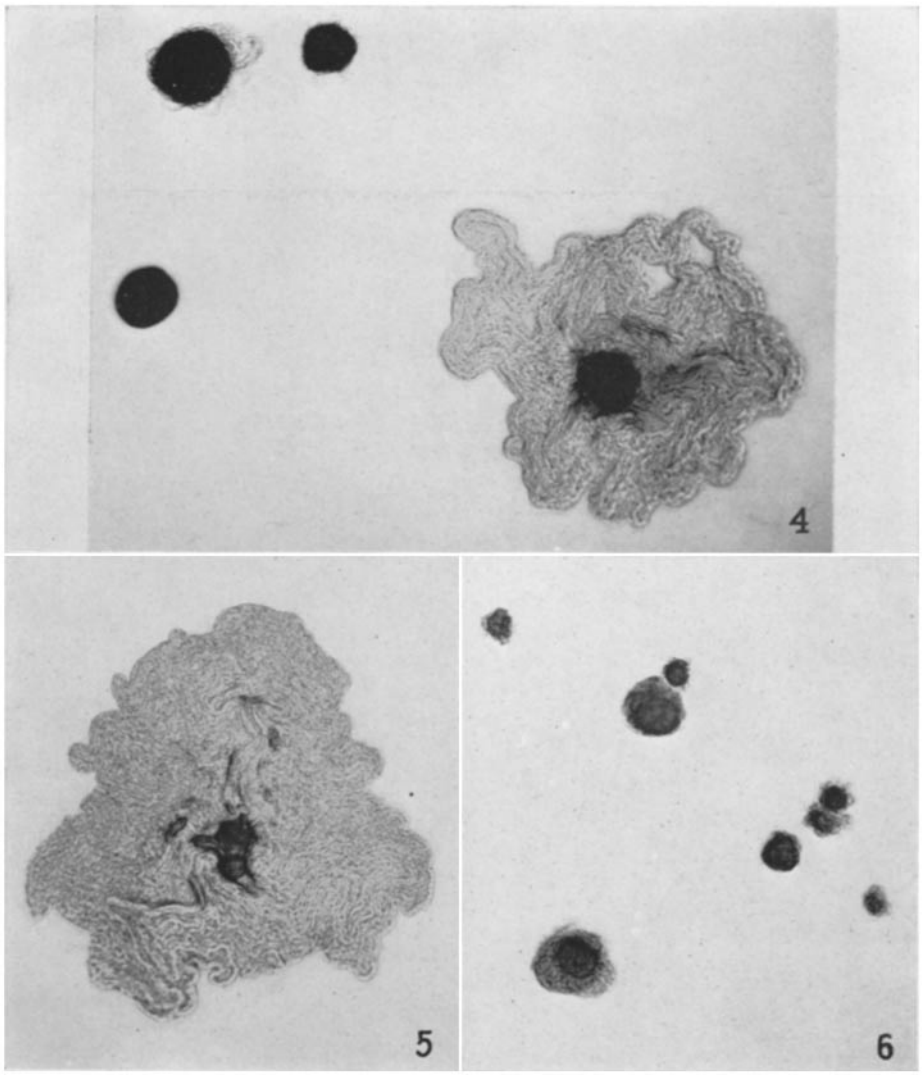
Colonial morphology of strain BCG III and of its substrains.

FIG. 4. Parent strain.

FIG. 5. Spreading substrain.

FIG. 6. Non-spreading substrain.

All colonies grown for 15 days on agar media containing oleic acid and bovine albumin. × 75.



(Suter and Dubos: Variability of BCG strains)

PLATE 49

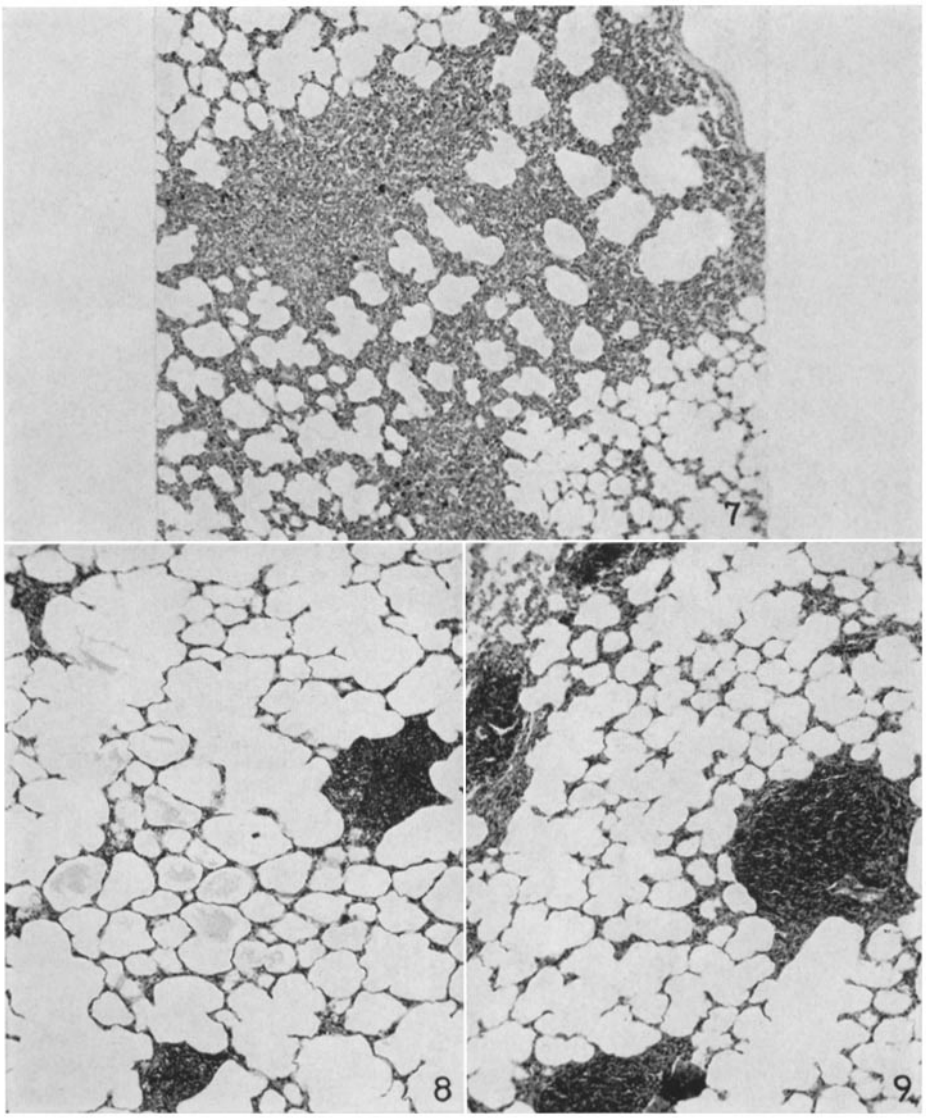
Sections of lungs of guinea pigs killed 12 weeks after intraperitoneal injection with 5 ml. of undiluted cultures of the three strains of BCG.

FIG. 7. Spreading substrain of BCG I.

FIG. 8. Spreading substrain of BCG II.

FIG. 9. Spreading substrain of BCG III.

The tissues were fixed in 4 per cent formaldehyde in physiological saline. The sections were stained with hematoxylin and eosin. × 77.



(Suter and Dubos: Variability of BCG strains)