The Problem of Standardization of BCG Vaccine*

JOSEPH D. ARONSON, M.D., F.A.P.H.A., AND PATRICIA SCHNEIDER

Henry Phipps Institute, University of Pennsylvania, and the Bureau of Tuberculosis Control, Commonwealth of Pennsylvania, Philadelphia, Pa.

ALTHOUGH BCG vaccine has been used widely as an immunizing agent for over 25 years, there remain many problems dealing with its preparation, preservation, and standardization.

The differences in results obtained by various investigators, with special reference to dose of vaccine, character of the local reaction and incidence and persistence of the post-vaccinal tuberculin reaction, make it advisable to study more precisely the technique used in the preparation of BCG vaccine and to develop methods for evaluating its potency.

CHARACTERISTICS OF THE BCG CULTURE

The culture, from which BCG stems, was isolated from the udder of a tuberculous cow by Nocard and was originally designated "lait Nocard." Its degree of virulence was such that the injection of 3 mg. intravenously was fatal to young calves. This culture was transplanted to potato soaked in beef bile containing 5 per cent glycerine and after 230 transplants on this medium, in the course of approximately 13 years, it had lost its ability to produce progressive tuberculosis in animals as susceptible as guinea pigs.

Studies have indicated that this attenuated strain now designated as BCG (B. Calmette-Guerin) retains all the characteristics of virulent strains of tubercle bacilli except their ability to produce progressive tuberculosis. Chargaff [1] has found that the BCG culture differs chemically from virulent human and bovine types of tubercle bacilli in that it contains a higher per cent of total lipids and chloroform-soluble waxes but the same per cent of phosphatides and polysaccharides as the virulent strains.

Calmette and Guerin [2] attributed the loss of virulence of the original culture to its repeated transfer to bile-containing medium. What effect, if any, bile may have on attenuating tubercle bacilli is a moot question. Steenken, Oatway, and Petroff [3] describe the dissociation of the H 37 strain, after it was grown on bile-potato medium for 4 months, into avirulent R colonies and virulent S colonies. Smithburn [4] found that a number of recently isolated human type tubercle bacilli were rather uniform in their pathogenicity. On the other hand, of 12 bovine strains studied, virulence was most marked in the most recently isolated strains, while some, but not all, of the older strains were so attenuated as to produce retrogressive lesions in both rabbits and guinea pigs. None of these cultures had been grown on bile medium. Frimodt-Möller [5] has reported the dissociation of two originally dysgonic, bovine strains grown on Löwenstein's and on Petroff's medium but not on bile-containing medium. From these two cul-

* Presented before the Laboratory Section of the American Public Health Association at the Seventy-seventh Annual Meeting in New York, N. Y., October 27, 1949.

tures, he isolated eugonic variants which resembled the BCG culture both as to virulence and growth characteristics. One of us (J.D.A) has observed that certain strains in our collection have gradually lost their virulence for the usual laboratory animals. The decrease of virulence varies with different strains and with some strains has been gradual, extending over a period of years. This we have observed with the Bovine III strain isolated in 1903 by Dr. Theobald Smith. On the other hand, a bovine strain isolated from the cervical lymph nodes and spleen of a horse, while highly virulent for rabbits and guinea pigs when first isolated, underwent a rapid attenuation after several transplantations on the usual glycerine agar medium. We have noticed that recently isolated avian strains have also shown a rapid loss of virulence for rabbits.

Our cultures of BCG have been obtained directly from the Pasteur Institute, Paris. In the experience of one of us (J.D.A.) since 1928, guinea pigs injected by various routes with from 1 to 20 mg. moist weight of the BCG culture have usually developed well defined local lesions, but in no instance has there been observed any evidence of progressive tuberculosis. While it is the consensus that the BCG culture is avirulent, and in that sense may be considered a virus fixé, its cultural characteristics vary as do those of other bacteria. Petroff, Branch, and Steenken [6] succeeded in dissociating the BCG culture into R colonies which were nonpathogenic for guinea pigs and S colonies which they claimed were pathogenic for these animals. While other investigators have confirmed the dissociation of this culture into the R and S colonies and into intermediate types of colonies, no one has corroborated the claim made by Petroff, Branch, and Steenken that the BCG culture was pathogenic for animals.

Over a period of years we have observed marked differences in the character of growth of BCG cultures. The rate, luxuriousness, and character of growth on potato medium soaked in either 5 per cent glycerine or Sauton medium varies within wide limits. These variations may be due to differences in the chemical and physical properties of the potato and its maturity or to the length of time of storage. In recent years our BCG culture has grown more rapidly and luxuriantly on Sauton medium than previously. Ordinarily in from 4 to 5 days the culture covers the surface of a 50 ml. flask, containing 25 ml. of Sauton medium, with a thin film which extends to the sides. Within the next 48 hours the film becomes thicker, the surface is wrinkled, waxy, dry, and has a yellow or tan color. The inoculated culture medium, which formerly developed a well marked yellow color, has in more recent years assumed a straw color or has remained colorless. From time to time we have observed definite opalescence in the culture medium after the culture has been grown for about 2 weeks. We have observed that the rate and luxuriousness of growth of the BCG culture is reduced if it is grown continuously on Sauton medium. Under these conditions there appear clumps of small, rough, rounded doughnut-like colonies with a central opening measuring about 3 to 5 ml. in diameter. This type of culture forms a granular suspension and when injected intracutaneously in man is followed by a significantly lower per cent of positive tuberculin reactions one year later than occurs with other cultures.

A third type of growth on Sauton medium, which we have designated hydrophilic, is characterized by the formation of a grayish white, wet, greasy appearing thick film which tends to be submerged and to form stalactites. This film falls to the bottom when the flask is shaken slightly. The hydrophilic type of culture contains 71 per cent of water in contrast to the 60 per cent found in

the waxy-like growth. Microscopically, the bacilli from the wet culture tend to be swollen, pale-staining, granular, and to contain numerous non-acid forms.

Because potato favors the rapid growth of tubercle bacilli, there is much to be said for the transfer of the BCG culture from the synthetic medium to potato. However, there is great variation in the luxuriousness of growth on different potatoes. Our observations indicate that the so-called Pennsylvania potato gives the most luxuriant growth, while the Maine potato and the Idaho potato give the least growth. We have also observed that, in general, growth is more uniform on potato during the summer months. Attempts to grow the BCG culture on carrots, rutabaga, and beets were unsuccessful. We have long discontinued the use of potato soaked in bile but have not observed any evidence of increased pathogenicity for guinea pigs.

Jensen[7] described significant differences in the virulence of two cultures of BCG obtained in 1927 and 1931 respectively from the Pasteur Institute, Paris. Vaccine prepared from the earlier culture produced large local lesions when injected, in man, in quantities of 0.01 or 0.001 mg., while the second culture gave rise only to a local nodule when 0.15 mg. was administered. Similarly the intracutaneous injection of varying amounts of these two cultures into guinea pigs indicated that the first culture was the more virulent. Neither culture, however, produced progressive tuberculosis in these animals. Jensen concluded that the continuous cultivation of the more attenuated culture on bile-potato increased its virulence to the level observed in the first culture, and that the use of bile-potato increases rather than decreases the virulence, as claimed by Calmette and Guerin.

Van Deinse[8] believes that the results obtained by Jensen can be attributed to the low vitality of the second culture, which was maintained continuously on Sauton medium, and that Jensen's results do not justify the conclusion that the bile-potato increased the virulence of the strain.

Böe[9] studied the virulence of cultures of BCG obtained from Sweden, Denmark, Norway, and the Pasteur Institute, Paris. He measured the virulence of the respective strains by determining the minimum amount of each culture which would produce a definite local lesion when injected intracutaneously into guinea pigs. The virulence of the 4 cultures was uniform despite the fact that the strain used in Sweden has been maintained exclusively on bile-potato for the past 20 years.

We have studied the virulence of the following 6 different strains of BCG obtained from different laboratories: BCG culture 186 received from Dr. J. Böe, Norway; culture 118 from Dr. K. Toderlund, State Serum Institute, Denmark; culture 793, series 2, Phipps Institute, Philadelphia, Pa., received from Pasteur Institute, Paris; culture 805 from Dr. Birkhaug, New York State Laboratory, Albany, N. Y.; culture 862 from Dr. S. R. Rosenthal, Tice Laboratory, Chicago, Ill.; and culture 425 from Dr. Wassen, Sweden. These cultures were transplanted to Sauton medium, and suspensions made from 7 day old cultures were injected intracutaneously into guinea pigs in tenfold dilutions ranging from 10^{-1} to 10^{-4} mg. of each culture. The rate of development, size, and character of local lesions were studied in each of the guinea pigs, all of which received the same dose of each culture. The results indicated that the character of the local lesions was not conspicuously different for the different strains but that in some guinea pigs local ulceration appeared earlier than in others.

The virulence of tubercle bacilli cannot be measured entirely on the basis of the minimum dose capable of producing local lesions, since due consideration

must also be given to the factor of natural resistance of the animal. Lewis and Loomis [10] have shown that the character of the local tuberculous ulcer produced by the intracutaneous injection of viable virulent tubercle bacilli into guinea pigs is related to the general resistance of the inbred family and is due to their constitutional differences. Dr. Max Lurie kindly placed at our disposal a rabbit from his tuberculosis-susceptible family and one from his tuberculosis-resistant family. Each rabbit was injected intracutaneously in the skin over the abdomen and in the skin over the back with 0.1 mg. of each of the 6 cultures of BCG. It was noted that in the susceptible rabbit the local inflammatory reaction, 24–48 hr. following the injection, was sharply defined and that the area of redness was extensive and deep red. This was most marked in the skin over the abdomen. In the resistant rabbit, on the other hand, the inflammatory reaction was less marked, diffuse, and pale red, and the edema gradually fused into the surrounding tissue. However, the inflammatory reactions elicited by the different cultures were not significantly different. With increasing time the differences in the appearance of the local lesions in the susceptible and resistant rabbits were more marked. In the susceptible rabbit the lesions were rounded, elevated, measured 10 to 12 mm. in diameter, and were raised from 3 to 4 mm. above the surrounding tissue. The rounded, marble shaped lesions were not adherent and moved freely in the surrounding tissues. On the other hand, in the resistant rabbit the lesions were flat and diffuse, measuring from 10 to 12 mm. in diameter, and raised about 1 mm. above the surrounding skin. Ulceration with discharge of caseous material was observed in the resistant rabbit 3 to 4 weeks after injection. Ulceration did not occur in the susceptible rabbit. In general, the lesions on the skin over the back of both animals were firmer and somewhat smaller than those noted over the abdomen.

While the number of animals used does not justify drawing final conclusions, we were impressed that over a long period of observation, the lesions produced by the BCG culture obtained from the different laboratories were not significantly different. Our observations are in agreement with those of Böe and indicate that the cultures of BCG obtained from 6 different laboratories are relatively uniform in their virulence, as determined by the intracutaneous injection of the cultures in both guinea pigs and rabbits. Some of the differences and irregularities which have been described in the literature may well be due to variations in the susceptibility of the animals used.

PREPARATION OF VACCINE

The procedure originally recommended by Calmette [2] has been followed implicitly by many laboratories. This consists of transplanting the culture which has been grown on potatoes soaked in beef bile containing 5 per cent glycerine, to potato soaked in 5 per cent glycerine. After 10 to 15 days' incubation, the film which has grown on the surface of the liquid is transferred to flasks of Sauton medium, where it is permitted to grow for from 8 to 10 days or until it covers the surface of the flask, when it is again transferred to a series of flasks containing Sauton medium. These flasks are incubated at 38° C. for from 20 to 25 days. The growth from this second generation on Sauton medium is collected under sterile conditions and the bacilli are suspended in 25 per cent Sauton medium to the desired concentration. According to Calmette the BCG culture when grown in flasks of 250 ml. capacity, containing 150 ml. of culture medium and under the conditions described, will yield 5 gm. of bacilli. The vaccine thus made should

be preserved in the ice box and used within 15 days.

It is customary to transfer the BCG culture after it has been grown for 10 generations on glycerine-potato to bile-potato. After two generations on bile-potato it is returned to glycerine-potato medium. The purpose of this alternation is, according to Calmette, to maintain the avirulence of the culture. Dr. Bretey [11] of the Pasteur Institute, Paris, has advised us that since 1939 the use of beef bile has been discontinued and that the BCG culture is now grown only on potato soaked in either 5 per cent glycerine or in Sauton medium.

The development of increased resistance following the administration of viable, attenuated organisms is dependent upon the initial rate of multiplication of the immunizing agent in the host. It would therefore seem logical to use a young actively growing culture of BCG for the preparation of vaccine rather than cultures which have grown for from 20 to 25 days, as recommended by Calmette. For many years we have used 7 to 10 day old cultures, for the preparation of BCG vaccine, and our studies indicate that when grown in Dubos medium, the BCG culture reaches its logarithmic peak of growth, from the 9th to 11th day.

For small scale production of vaccine, we collect the bacilli from a 7 to 10 day old culture on Sauton medium by filtering the culture through a sintered glass filter of medium porosity. The bacillary mass is collected from the sintered glass plate and transferred to sterile filter paper, where the excess moisture is removed. The bacillary mass is transferred to a piece of sterile platinum foil and the weight is determined by difference. The weighed mass is placed in a sterilized, heavy walled, globular, glass flask containing stainless steel or Monel metal balls. It is ground at first into a paste by the addition of a few drops of the diluent, after which the diluent is gradually added to bring the vaccine to the desired concentration. In addition to determining the weight of the bacillary mass, it has been our practice to determine the opacity of each lot of vaccine with the Klett-Summerson colorimeter. We have obtained a better correlation between opacity and the viability of the vaccine, as determined by the minimum amount of the vaccine capable of initiating growth in Dubos medium, than by using the weight of the bacilli in relation to viability. This difference in correlation is due to the marked variation in the size and water content of the bacilli. With a reading of 40 to 59 on the Klett-Summerson colorimeter, growth in Dubos medium was obtained with a dilution of 10^{-6} mg. in 55.5 per cent of 18 lots of vaccine, while with a reading of 60 to 79, growth was obtained in a dilution of 10^{-6} mg. in 62.5 per cent of 8 samples.

Because of the marked variation in the size of different strains of avian tubercle bacilli, Van Deinse and Hoogheimster [12] concluded that the number of bacilli, rather than the weight, should be used in preparing bacillary suspensions. We have also observed marked variation, not only in the per cent of water present in different cultures, but also in the size of the bacilli from the same strain, when samples of organism were measured from different types of growth.

We have found that the vaccine prepared by grinding the bacilli for a long period of time with stainless steel balls may have a gray or black color. That this is due to some interaction between the metal and the bacilli is suggested by the fact that none of the diluents used, such as Sauton medium, physiological salt solution, or phosphate buffer pH 7.2 are discolored when the steel balls are permitted to remain in these solutions for long periods of time or are sterilized in these solutions at 15 lb. pressure for 20 to 30 min. It is inadvisable to use glass beads or mullite balls

for making the bacillary suspension since there exists the danger of their chipping and leaving particles of glass in the vaccine. For these reasons we have been using Monel metal balls.

We have also investigated the effect of grinding on the viability, staining and morphology of the bacilli. Bacillary suspensions of 40 mg. per ml. were ground for from 5 min. to 1 hr. with either glass beads or stainless steel balls. Samples were removed after grinding for 5 min. and at 15 min. periods thereafter for 1 hr. It was found that there was no change in the tinctorial or morphological characteristics after grinding the bacilli with glass beads for 30 min. Samples examined after 45 min. to 60 min. grinding retained their tinctorial character, but the cells were definitely shrunken. Samples of vaccine ground for from 5 to 15 min. with stainless steel balls showed no change in the staining or morphology of the organisms. However, samples collected after 30 min. grinding show marked reduction of the number of acid-fast organisms and increasing numbers of non-acid-fast bacilli. After grinding for 45 or 60 min. with the stainless steel balls, the majority of the organisms have disintegrated and few acid-fast bacilli remain.

The viability of the bacteria after grinding for various periods of time with either the glass beads or steel balls was determined by adding tenfold dilutions of the ground bacillary suspensions to Dubos medium and incubating the culture for 30 days. The minimal amount of the bacillary suspension capable of initiating growth after grinding with either glass beads or steel balls is shown in Table 1.

It will be noted that grinding with stainless steel balls reduces the number of viable bacilli somewhat more rapidly than is the case with glass beads. It was also noted that there was a lag in the rate of growth of the bacillary mass, ground with the steel balls, over that observed with suspensions prepared by grinding with glass beads. The reduction of bacilli may be due to injury of the cell by grinding and in the case of the stainless steel balls the metal may have an oligodynamic action.

TABLE 1

Effect of Grinding on the Viability of BCG Vaccine

Minimal Amount in MG Capable of Initiating Growth in Dubos Medium after Grinding for Variable Periods of Time

	Time of Grinding in Minutes				
	5	15	30	45	60
Glass beads	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-3}
Stainless steel balls	10^{-5}	10^{-5}	10^{-3}	10^{-3}	10^{-3}

THE TUBERCULIN REACTION FOLLOWING BCG VACCINATION

To estimate the relative importance of some factors involved in the preparation of BCG vaccine, Aronson, Saylor, and Parr [13] studied the incidence of positive tuberculin reactions following the administration of 13 different lots of BCG vaccine, prepared under different conditions. A group of 1,565 Indians, ranging in age from less than 1 year to 20 years, who failed to react to the intracutaneous injection of 0.005 mg. of PPD tuberculin were injected intracutaneously with either 0.1 or 0.15 mg. of freshly prepared BCG vaccine. The different lots of vaccine used were prepared from BCG cultures grown on Sauton medium at 38° C. for from 7 to 30 days, and which had been grown on bile-free medium for from 2 to 7 generations. These preparations were generally used within the first 24 hr. and only a few persons received vaccine as old as 72 hr. The tuberculin test was repeated annually on these persons, using an initial dose of 0.00002 mg. PPD, and — if negative after 48 hr., the test was repeated with 0.005 mg. PPD and read after another 48 hr. From the beginning of this study until 1942 the PPD prepared by Dr. Florence Seibert, Henry Phipps Institute,[14] by precipitation with

trichloracetic acid was used and after that PPDS prepared by Dr. Seibert by precipitating the tuberculin with ammonium sulphate[15] was used. The PPD tuberculin was diluted each day from the concentrated solution and the injections and reactions were read by the same persons. This investigation was conducted for 11 years in some areas and 9 and 10 years in other areas. Throughout this time 1,545 persons or 98.7 per cent of the original group of 1,565, were followed. During the annual examinations, never less than 69 per cent of the original group were retested and for the first 5 years more than 90 per cent were retested each year.

In summarizing the reactions following vaccination we have grouped those which were persistently positive to 0.00002 mg. PPD or to 0.005 mg. PPD with those who reacted sometimes to one dose and sometimes to the other dose but who were at all times positive. The second group contains those who were positive one year after vaccination and who after that were usually positive, with occasional negative or doubtful reactions to 0.005 mg. PPD. Of individuals who were tuberculin-negative or doubtful one year after vaccination, there were those who never became positive, those who subsequently became and remained positive, and those who became positive and occasionally were negative or doubtful to 0.005 mg. PPD. It will be observed from Table 2 that, within the limits of this study, the incidence of the tuberculin reaction, 1 year after vaccination, is unrelated to the age of the culture, the number of generations through which the culture has been grown on bile-free medium, or the dose of vaccine used. Similarly, the persistence of the tuberculin reaction during the 9 to 11 year period of observation bears no relationship to the factors mentioned above nor to the incidence of the tuberculin reaction 1 year after vaccination. Examination of Table 2 shows that from 95 to 100 per cent of those who received vaccine lots 3, 4, 5, 6, 10, 11, 12 or 13 were tuberculin-positive 1 year later, and that the tuberculin reaction persisted throughout the period of this study in from 87 to 100 per cent of these cases. Of those who were injected with vaccine lots 8 or 9, the tuberculin reaction was positive 1 year after vaccination in 61.8 and 88.1 per cent of cases respectively and a significant per cent of these cases remained positive with occasional negative or doubtful reactions during this study. A third group, injected with vaccine lots 1, 2 or 7, present a problem of special interest. Vaccine lots 1 and 2 were used in 1936 and lot 7 in 1937. It will be noted that while the tuberculin reaction was present one year after vaccination, in 92.6 and 94.7 per cent of those injected with lots 1 and 2 respectively, in the course of 11 years of observation, a persistent tuberculin reaction was obtained in 57.7 and 66 per cent of those who received lots 1 and 2 respectively, while 35.3 and 26.8 per cent respectively gave positive tuberculin reactions interspersed at irregular intervals with negative or doubtful reactions. Similarly although 93.5 per cent of those who received vaccine lot 7 reacted to tuberculin 1 year after vaccination, a persistent tuberculin reaction over a period of 10 years observation occurred only in 69 per cent, while 23.7 per cent gave a tuberculin reaction which fluctuated from positive to negative or doubtful. It will be noted that vaccine lots 1, 2 and 7 were all used on the Pima Agency, Arizona. This instability of the tuberculin reaction among those living on the Pima Agency has been noted since the early years of our study; it was even higher among an equal number of unvaccinated controls who became positive in the course of succeeding years. The instability of the tuberculin reaction as related to different geographical areas will be reported on at a later date.

TABLE 2

Duration and Character of Tuberculin Reaction to Different Preparations of BCG Vaccine

Agency	*Lot No. of Vaccine*	*Age of Culture in Days*	*Generations after Bile*	*Dose in Mg.*	*No. Vaccinated*	*No. of Yrs. Observed*	*Total Per cent of Reactions to Tuberculin during Period of Observation*					
							Pos. 1 Yr. Later	*Persistently Pos.*	*Pos. with Occas. Neg. or Doubt.*	*Persistently Neg. or Doubt.*	*Neg. or Doubt. to Persistently Pos.*	*Neg. or Doubt. with Occas. Pos.*
Pima	1	14	7	0.15	88	11	92.6	57.7	35.3	3.5	3.5	0.0
Pima	2	21	7	0.15	169	11	94.7	66.0	26.8	0.0	5.9	1.2
Wind River	3	30	3	0.15	101	11	95.6	87.6	7.2	0.0	5.2	0.0
Wind River	4	14	3	0.15	115	11	99.1	92.3	6.9	0.0	0.8	0.0
Wind River	5	7	6	0.1	12	11	100.0	100.0	0.0	0.0	0.0	0.0
Turtle Mountain	6	13	7	0.1	170	11	98.2	85.1	13.1	0.0	1.2	0.6
Pima	7	16	2	0.15	97	10	93.5	69.0	23.7	1.1	2.1	4.1
Rosebud	8	17	4	0.15	127	9	61.8	41.8	19.7	13.4	19.4	5.5
Rosebud	9	17	5	0.15	164	9	88.1	71.4	16.4	4.2	5.5	2.5
Rosebud	10	21	7	0.15	10	9	100.0	100.0	0.0	0.0	0.0	0.0
Alaska	11	21	2	0.1	268	9	98.8	94.5	3.7	0.0	1.5	0.3
Alaska	12	19	3	0.1	29	9	100.0	100.0	0.0	0.0	0.0	0.0
Alaska	13	10	4	0.1	215	9	100.0	97.7	2.3	0.0	0.0	0.0
Total					1565		93.3	79.9	13.0	1.8	3.9	1.8

STABILITY OF BCG VACCINE

The effectiveness of BCG vaccine in increasing resistance is dependent on the number of viable bacilli. The relationship of the viability of the organism to its antigenic property and the lability of this antigenicity has been demonstrated by Smithburn and Lavin.[16] These investigators found that tubercle bacilli in a concentration of 1 mg. per ml., exposed to ultraviolet light in the range of 2537 Å for 9 min. became attenuated but remained viable and induced resistance to reinfection when injected into guinea pigs. On the other hand, cultures exposed for 10 min. to this range of light were killed and lost their immunizing properties.

One of the major drawbacks to the widespread use of BCG vaccine has been the rapid and marked decrease in the number of viable bacilli when they are suspended in different liquid menstruums. Because of this, BCG vaccine must be used within several days after it is prepared. This does not permit a determination of avirulence, tuberculogenic property, number of viable bacilli or its protective value before use.

Jensen[7] has reported a marked decrease in the virulence and number of viable bacilli after storing BCG vaccine for 2, 5, and 11 days at 2° and 18° C. Jensen attributed this decrease in virulence and viability to the presence of copper in the water used to suspend the organisms. We have investigated the bactericidal property of copper on BCG vaccine and have found that the addition of copper sulphate in a final concentration of 1 to 10,000,000 to BCG vaccine suspended in a phosphate buffer pH 7.2 had no inhibitory effect on the vaccine. The water used in making up the buffer was distilled in glass. The addition of copper sulphate in a final concentration of 1 to 100,000 increased the rate and luxuriousness of growth of the organism.

It has been our experience that BCG vaccine, prepared in a concentration of 1 mg. per ml. from 7 to 10 day old cultures on Sauton medium, shows a significant decrease in the number of viable organisms kept from 5 to 7 days at either 4° or 20° C. For this reason we recommend that BCG be used within 4 to 5 days after its preparation. When the concentration of organisms is increased to 2 or 3 mg. per ml. a larger number remain viable but a correspondingly larger number of dead bacilli are also present, which may give rise to a more severe local reaction.

The effect of age on BCG vaccine can be further demonstrated by the decrease in its tuberculogenic property. The intracutaneous injection of 0.1, 0.01 and 0.001 mg. of fresh BCG vaccine into guinea pigs gives rise to a local ulcer or tubercle, while 0.0001 mg. of the vaccine does not usually induce a demonstrable lesion. On the other hand, when the same amounts of a 10 day old vaccine are injected, a local lesion is usually obtained only with 0.1 mg. and slight or no lesion with the smaller amounts.

The number and viability of bacteria is customarily determined by inoculating varying amounts of the organisms into suitable culture mediums. Since the growth of the tubercle bacillus requires from 3 to 4 weeks' incubation, this method is impractical in standardizing the rapidly deteriorating BCG vaccine. We have therefore studied the relationship of oxygen uptake of the bacteria to their viability, realizing that a gradient exists between the fully active living cells and the dying and dead cells. The oxygen uptake of samples of BCG vaccine has been determined by means of the Barcroft-Warburg apparatus and by means of the Scholander[17] microrespirometer. Preliminary experiments, using varying quantities of heat-killed and living BCG vaccine, indicated a definite relationship between oxygen uptake and the per cent of living cells present in the mixture.

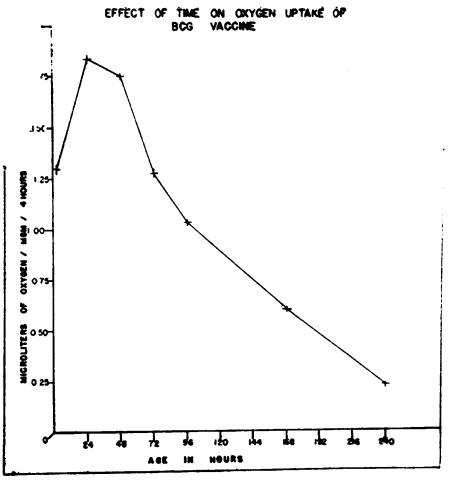
The determination of oxygen uptake

of the BCG vaccine was carried out in the Barcroft-Warburg apparatus by washing the bacteria on a sintered glass funnel and suspending them in a concentration of 50 mg., moist weight, per ml. in phosphate buffer pH 7.0, after which readings were taken at 15 min. intervals for a period of 4 hr. It was found that the oxygen uptake of the BCG vaccine decreased significantly with increasing age of the vaccine. Similar studies were conducted using the Scholander microrespirometer. The BCG vaccine in a concentration of 1 mg. per ml. was stored at 4° C. and the oxygen uptake was determined on samples of the same suspension at varying times after its preparation. The original sample was centrifuged, washed 3 times with phosphate buffer pH 7 and resuspended in fresh buffer solution in a concentration of 30 mg. per ml. Of this concentrated material, 0.066 ml. containing 2 mg. of the bacilli was transferred to a micro flask and a drop of 20 per cent potassium hydroxide was added to the small side arm. The micro flask was attached to the arm of the respirometer and the unit was kept in the water bath at 37.5° C. The oxygen uptake was determined at intervals of 30 min. for a total of 4 hr. The results of such an evaluation are presented graphically in Figure 1.

It will be observed from this chart that cell respiration as measured by its oxygen uptake decreases rapidly with increasing age of the vaccine. The results of these studies suggest the usefulness of this method for determining expeditiously the viability of the cells in the BCG vaccine.

Because of the instability of the BCG vaccine, prepared with physiological saline or phosphate buffer pH 7.2, we have investigated the usefulness of other solutions in stabilizing the vaccine. The different preparations which we have tried and the results obtained are presented in Table 3.

It will be observed that, with the exception of dextrin, none of the solutions tested was able to maintain the viability of the organisms for as long as 6 days.



Because of the instability of suspensions of BCG vaccine, the possibility of preserving the vaccine by lyophilization over anhydrous calcium sulphate was investigated. The BCG vaccine was suspended in gelatins varying in their isoelectric point, sodium ammonium pectate, certain carbohydrates, and in glutamic, malic, and succinic acids. Cultures of the vaccine made immediately after lyophilization showed a marked decrease in the number of viable organisms.

We have investigated the relative value of different culture mediums as to their ability to initiate growth with small amounts of inoculum. We have found that of the solid medium studied, the Trudeau Committee medium consisting of egg yolk, potato flour, and glycerine gave the best results. Of the liquid synthetic mediums, we have found growth appears earlier and can be initiated with a smaller amount in Youmans medium than in Dubos medium containing Tween 80.

TABLE 3

Effect of Age on Viability of BCG Vaccine Suspended in 1 Mg. per Ml. in Different Solutions

	Minimal Amount of BCG Vaccine Initiating Growth in Dubos Medium						
	Age of Vaccine						
Solution	*3 hours*	*3 days*	*6 days*	*10 days*	*12 days*	*22 days*	*25 days*
Physiological saline	10^{-7} mg.	10^{-5} mg.	10^{-2} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.	No growth
Ringer solution	10^{-5} mg.	10^{-2} mg.	10^{-2} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.	No growth
Phosphate buffer pH 7.2	10^{-6} mg.	10^{-6} mg.	10^{-2} mg.	10^{-2} mg.	10^{-2} mg.	10^{-1} mg.	10^{-1} mg.
Sauton medium 1:3 dilution	10^{-6} mg.	10^{-6} mg.	10^{-2} mg.	10^{-1} mg.	10^{-2} mg.	10^{-1} mg.	No growth
Thiourea 0.5%	10^{-6} mg.	10^{-4} mg.	No growth	No growth	No growth	No growth	No growth
Horse serum 10% in saline	10^{-3} mg.	10^{-3} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.
Milk 10%	10^{-5} mg.	10^{-4} mg.	10^{-3} mg.	10^{-2} mg.	10^{-2} mg.	No growth	No growth
Bovine Albumin 5% (Fraction V)	10^{-7} mg.	10^{-6} mg.	10^{-2} mg.	10^{-3} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.
Pectin 1.0%	10^{-7} mg.	10^{-6} mg.	10^{-3} mg.	10^{-2} mg.	10^{-2} mg.	10^{-1} mg.	10^{-1} mg.
Mannitol 1.0%	10^{-7} mg.	10^{-6} mg.	10^{-3} mg.	10^{-2} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.
Dextrin 1.0%	10^{-4} m	10^{-3} mg.	10^{-4} mg.	10^{-2} mg.	10^{-1} mg.	10^{-1} mg.	10^{-1} mg.
Ascorbic acid 0.5%	10^{-8} mg.	10^{-7} mg.	10^{-3} mg.	10^{-2} mg.	10^{-1} mg.	No growth	No growth
Cystine, saturated solution	10^{-4} mg.	10^{-5} mg.	10^{-3} mg.	10^{-2} mg.	10^{-2} mg.	10^{-3} mg.	10^{-1} mg.
Cysteine 0.5%	10^{-5} mg.	10^{-5} mg.	10^{-3} mg.	10^{-1} mg.	10^{-1} mg.	No growth	No growth

EVALUATION OF BCG VACCINE ON BASIS OF PROTECTION

Although it is true that the administration of large amounts of tubercle bacilli, living or dead, or fractions of the tubercle bacillus, may result in the production of humoral antibodies, there is no acceptable evidence that such antibodies play any role in acquired resistance to tuberculosis. The relatively small amount of organisms administered to man in BCG vaccine is insufficient to elicit humoral antibodies, but is capable of inducing allergy as determined by means of the tuberculin reaction. The development of allergy cannot, however, be accepted as definite evidence of resistance to reinfection.

The final proof of the efficacy of an immunizing agent is its ability to protect against a challenge dose of the specific virulent infecting agent. At the present time the effectiveness of BCG vaccine is measured by its ability to induce a positive tuberculin reaction in man.

In order to estimate the protective value of BCG vaccine we have determined its effectiveness in protecting mice against a challenge dose of virulent tubercle bacilli. Mice weighing from 18 to 20 gm. were injected intraperitoneally or subcutaneously with 0.1 mg. of BCG vaccine in a volume of 0.1 per ml. One month after receiving the vaccine the mice were injected intravenously, in the most prominent caudal vein, with varying amounts of a 7 to 10 day old culture of the virulent Ravenel bovine type of tubercle bacillus grown in Dubos medium containing Tween 80. With this dose the unvaccinated animals die in from 2 to 4 weeks and at necropsy there is noted extensive confluent tuberculous bronchopneumonia. The lungs are markedly congested and edematous in those dying early, while in those dying later there are observed numerous, extensive, caseous areas. The spleen is swollen and weighs from 3 to 4 times that of the normal. Many of the BCG vaccinated mice receiving the larger challenge dose of virulent bacilli also show extensive tuberculosis of the lungs but with the smaller doses many of the BCG vaccinated animals survive and show no gross lesions of tuberculosis.

We have found that the intravenous administration of the challenge dose

gives the most uniform results. While the intranasal instillation of the virulent tubercle bacilli in partially anesthetized mice gives a high per cent of pulmonary lesions, the method involves some risk to the investigator and the results are not as uniform as are those obtained with the intravenous route. The intracerebral injection of the challenge dose gives highly irregular results, while the intraperitoneally injection of the challenge culture in Dubos medium or in mucin fails to produce pulmonary lesions. Studies are in progress to determine the optimum dose of BCG vaccine which will protect against a challenge dose of virulent tubercle bacilli, with the hope that such a method will permit the evaluation and standardization of BCG vaccine.

SUMMARY

1. While the colonial and antigenic characteristics of the BCG culture may vary, we have not observed any evidence of increased virulence.
2. Six cultures of BCG obtained from different laboratories both in the United States and abroad are uniformly avirulent, and there is no evidence that the cultivation on bile-containing medium increases the virulence of the culture.
3. The nature of the local lesions in animals following administration of the BCG culture varies with the natural resistance of the animals.
4. The persistence of the tuberculin reaction in man, following the intracutaneous injection of BCG vaccine, is not correlated with the age of the culture nor with the number of generations for which the culture has been maintained on bile-free medium.
5. The instability of the tuberculin reaction following BCG vaccination is correlated to some degree with geographical location.
6. The viability of the organisms in BCG vaccine after 5 to 7 days decreases rapidly and continues to decrease with increasing age, as indicated by cultural methods, tuberculogenic property, and oxygen consumption.
7. Lyophilization reduces immediately the number of viable organisms present in BCG vaccine.
8. Mice can be protected by BCG vaccine against a challenge dose of virulent tubercle bacilli.
9. The universal use of BCG vaccine and its manufacture by commercial organizations should depend on stabilization of the vaccine and development of standards based on protection tests with suitable animals.

NOTE—We greatfully acknowledge the technical assistance of Mrs. Mary Ann Stewart and Mr. Walter Flagg.

BIBLIOGRAPHY

1. Chargaff, E. Über die Lipoide des Bacillus Calmette-Guerin (BCG). *Ztschr. f. physiol. Chem.* 217, 115, 1933.
2. Calmette, A. *L'infection Bacillaire et la Tuberculose.* Paris, Masson et Cie, 1936.
3. Steenken, W., Jr., Oatway, W. H., and Petroff, S. A. Biological Studies of the Tubercle Bacillus. III. Dissociation and Pathogenicity of the R and S Variants of the Human Tubercle Bacillus (H37). *J. Exper. Med.* 60:515, 1934.
4. Smithburn, K. C. The Colony Morphology of Tubercle Bacilli. *J. Exper. Med.* 62:645, 1935.
5. Frimodt-Möller, J. Dissociation of Tubercle Bacilli. *Acta tuberc. Scandinav.* Suppl 11, 1939.
6. Petroff, S. A., Branch, A., and Steenken, W., Jr. Microbic dissociation 111. (Bacillus Calmette-Guerin). *Proc. Soc. Exper. Biol. & Med.* 25:14, 1927.
7. Jensen, K. A. Practice of the Calmette Vaccination. *Acta tuberc. Scandinav.* 20, 1, 1946.
8. Van Deinse, F. The Preservation of the BCG Strain. *Am. Rev. Tuberc.* 58:571, 1948.
9. Böe, J. Variations in the Virulence of BCG. *Acta tuberc. Scandinav.* 22:125, 1948.
10. Lewis, P. A., and Loomis, D. Ulcerative Types as Determined by Inheritance and as Related to Natural Resistance against Tuberculosis. *J. Exper. Med.* 43:449, 1928.
11. Bretey J. Personal Communication.
12. Van Deinse, F., and Hooghiemster, E. Contribution a l'etude de la Morphologie des Bacilles Tuberculeux du Type Aviaire, *Compt. rend Soc. de biol.* 123, 243, 1938.
13. Aronson, J. D., Saylor, R. M., and Parr, E. T. BCG Vaccine Its Preparation and the Local Reaction to Its Injection. *Am. Rev. Tuberc.* 42:651, 1940.
14. Seibert, F. B. The Isolation and Properties of the Purified Protein, Derivative of Tuberculin. *Am. Rev. Tuberc.* 30:713, 1934.
15. Seibert, F. B., and Glenn, J. T. Tuberculin Purified, Protein Derivative, Preparation and Analyses of a Large Quantity for Standard. *Am. Rev. Tuberc.* 44:9, 1941.
16. Smithburn, K. C., and Lavin, S. D. The Effect of Ultraviolet Radiation on Tubercle Bacilli. *Am. Rev. Tuberc.* 39:782, 1939.
17. Scholander, P. F. *Rev. Scient. Instruments* 13:32, 1942.