PRODUCTION OF BCG VACCINE IN A LIQUID MEDIUM CONTAINING TWEEN 80 AND A SOLUBLE FRACTION OF HEATED HUMAN SERUM

II. Antigenicity of the Culture after Various Periods of Storage

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(Received for publication, November 17, 1949)

As shown in the preceding paper, it is possible to obtain submerged, diffuse growth of strains of BCG, by cultivating them for 8 days at 37°C. in a liquid medium containing the wetting agent Tween 80 and the soluble fraction of heated human serum. A high percentage of the organisms present in these cultures can survive prolonged periods of storage at ice box and incubator temperatures (1).

The present paper deals with the antigenic response of guinea pigs to the injection of cultures grown under these conditions and stored at 4°C. for periods as long as 6 weeks.

EXPERIMENTAL

The culture media, the bacteriological techniques, and the strain of BCG used in the present study have been described in the preceding paper (1).

The guinea pigs were female, smooth haired albino animals of the Rockefeller Institute stock. They came from a colony selected by Dr. M. W. Chase (2) on the basis of high susceptibility to sensitization with 2:4 dinitrochlorobenzene. The animals were pen-inbred, only the bucks being selected for susceptibility to sensitization.[1] The stock has been rendered and maintained free of carriers of group C streptococci by skin testing.

Throughout the experiment, the guinea pigs were kept in the same room, which was constantly irradiated with a General Electric germicidal lamp (2537 λ wave length). They were housed in groups of two or three per cage and were fed a diet of hay, oats, and cabbage.

At the beginning of the study, on March 9, 1949, they were tested for sensitivity to tuberculin by the intracutaneous injection of 0.005 mg. of PPD (Sharp & Dohme). None of them gave a positive reaction.

Effect of Dose of BCG on the Development of Tuberculin Allergy.—The purpose of the following experiment was to determine the minimal number of units of BCG capable of rendering guinea pigs allergic to tuberculin.

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[1] We are informed by Dr. M. W. Chase that thsee animals, although uniform with reference to their susceptibility to sensitization with 2:4 dinitrochlorobenzene, are heterogeneous in their response to sensitization to tuderculin.

Guinea pigs weighing approximately 300 gm. were injected subcutaneously (in the groin) or intracutaneously with various dilutions in 0.1 per cent albumin of a culture of BCG grown in the liquid medium containing Tween 80 and the soluble fraction of heated human serum. The number of viable units in the culture was determined by plating serial dilutions of

TABLE I

Effect of Injection of Different Doses of Living BCG on the Production of Tuberculin Allergy in Guinea Pigs

No. of viable units of BCG injected	Route of injection	Tuberculin test			
		Material used	Time interval between injection of BCG and tuberculin test	Result at 48 hrs.	
				Diameter of erythema	Remarks
			wks.	*mm.* × *mm.*	
400,000	Subcutaneous	Old tuberculin (dilution 1/1000)	5	17 × 15	*Cocarde*
				12 × 12	Necrotic center
				12 × 10	—
4,000	"	" "	"	13 × 12	*Cocarde*
				15 × 12	Necrotic center
				10 × 10	" "
40	"	" "	"	15 × 10	" "
				10 × 8	—
"0.4"	"	" "	"	0	—
				0	—
1,100,000	Intracutaneous	PPD 0.0005 mg.	6	17 × 15	*Cocarde*
				14 × 14	Necrotic center
11	"	" " "	"	11 × 9	" "
				8 × 8	—
"1"	"	PPD 0.005 mg.	"	0	—
				0	—

it on oleic acid–bovine albumin agar (3, 4). Five to 6 weeks later, the animals were tested for sensitivity to tuberculin by the injection of either old tuberculin or PPD (Sharp & Dohme) (Table I).

It is apparent from the results presented in Table I that guinea pigs can be sensitized to tuberculin by the injection—either subcutaneous or intracutaneous—of less than 100 viable BCG units. Moreover the tuberculin reactions

elicited by the injection of either O.T. or PPD 6 weeks after vaccination were approximately of the same intensity with all doses of vaccine except with those containing very few viable units.

Local Lesions Produced by BCG.—As will be pointed out in a later section of this report (see Table IV), the intracutaneous injection of 0.05 cc. of BCG cultures grown in the Tween-serum filtrate medium resulted in lesions larger than those produced by the intracutaneous injection of 0.05 cc. of a BCG vaccine prepared by the standard technique (5). An experiment was instituted to test whether this difference was due to a greater intrinsic virulence of the former

TABLE II

Lesions Arising in Guinea Pigs Following the Intracutaneous Injection of Various Doses of Living BCG

Substrain of BCG*	No. of viable units per cc. of undiluted subculture	Guinea pig No.	Lesion arising at sites of injection of various doses of BCG culture						
			Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
			mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
Culture carried for 2 years in Tween-albumin medium	25×10^6	2-07	10‡ Ulcer	7	4	2	Tiny nodule	Tiny nodule	—
		38-46	10 Ulcer	6 Ulcer	"	"	" "	" "	—
First transfer from sample of standard BCG vaccine	11×10^6	2-07	9 Ulcer	6	3	3	" "	2 (erythema only)	—
		38-46	9 Ulcer	5 Ulcer	4	3	" "	" "	—

* Both substrains subcultured in Tween-serum filtrate medium; the numbers of viable units reported here are within the range of errors of the enumeration technique as used in this experiment.

‡ Diameter in millimeters of the red nodule at the infection site 7 days after injection; examination for ulcers made 14 days after injection.

cultures, or to the fact that they contained more viable organisms than the standard vaccine.

The two substrains of BCG (*i.e.* the one carried for 2 years in our laboratory in Tween-bovine albumin medium, and one supplied for this particular experiment in the form of a sample of standard vaccine by Dr. Aronson) were inoculated into the liquid medium containing Tween and human serum filtrate. After 8 days' incubation at 37°C., 0.05 cc. of serial tenfold dilutions of each subculture was inoculated intracutaneously into two guinea pigs, one series of dilutions on either side. The sizes of the resulting lesions, observed 1 week and 2 weeks after inoculation, are recorded in Table II.

The results presented in Table II show that the culture of the BCG strain maintained for 2 years in the Tween-bovine albumin medium and the culture

recovered from a new sample of standard BCG vaccine, produced lesions of indistinguishable severity when comparable dilutions of growths in Tween-serum-filtrate medium were injected intracutaneously into normal guinea pigs.

Antigenic Response of Guinea Pigs to Vaccination with Various Preparations of BCG.—The following experiment was designed to determine the effect of storage at 4°C. on the antigenic efficacy in guinea pigs of cultures of BCG which had grown diffusely in a liquid medium containing Tween 80 and a filtrate of heated human serum. A BCG vaccine prepared by the conventional technique, and used within 48 hours after its preparation, served as a standard of reference.[2]

Half the guinea pigs used in this experiment were 4 to 5 months old and had weights of the order of 500 gm. at the beginning of the experiment; the others were 2½ to 3 months old and weighed approximately 300 gm. Equal numbers of animals of the two age groups were assigned by random selection to each of the experimental groups. Vaccination with the bacterial suspensions (or uninoculated medium in the control animals) was administered on March 15, 1949. It consisted of two simultaneous intracutaneous inoculations, one over each shoulder, each of 0.05 cc. of the standard vaccine, or undiluted culture, or uninoculated medium.

A tuberculin test was made on April 26, 6 weeks after vaccination, 0.0005 mg. tuberculin PPD (Sharp and Dohme) being used for the vaccinated animals and 0.005 mg. for the controls.

The challenge infection was carried out on May 6th by injecting subcutaneously over the sternum 0.1 cc. of an undiluted culture in Tween-bovine albumin medium, of virulent human tubercle bacilli of the Amerzanga strain;[3] the inoculum contained 13 million viable bacterial units (single cell or small clumps). Some of the guinea pigs which had received the BCG vaccines were kept uninfected to serve as controls on the virulence of the vaccines.

At weekly intervals from the beginning of the experiment, all animals were weighed on a spring balance, weights being read to the nearest 5 gm. They were inspected at the same time to detect the occurrence and measure the extent of the local skin lesions produced by the injections of BCG and, later, of virulent tubercle bacilli. The size of the axillary and inguinal lymph nodes was also estimated by palpation. Necropsies were performed as soon as possible after death on all animals that died during the course of the experiment, and sections of the liver, spleen, and lungs were made and stained with hematoxylin and eosin, and by the Ziehl-Neelsen technique. The experiment was concluded on July 15, approximately 10 weeks after infection with the virulent tubercle bacilli. Ten of the sixteen unvaccinated animals had already died, and the survivors of all groups were killed with chloroform and necropsied during the next 4 days. Sections were made of lesions not obviously tuberculous, as well as of the lungs, liver, and spleen of all animals that had received only BCG vaccine (groups IB and IIB).

The details of the subdivision of guinea pigs into several groups are shown in Table III, and some of the results of the vaccination and challenge infection in Table IV.

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[2] This sample of standard vaccine, prepared at the Henry Phipps Institute in Philadelphia, was kindly supplied by Dr. J. Aronson. It was injected into the animals immediately upon receipt, the day after it had been issued from the Henry Phipps Institute.

[3] This strain was kindly supplied by Dr. J. Aronson.

Thirteen animals died between commencement of the experiment on March 3 and its completion on July 14. Ten of these were non-immunized infected animals (group V), and they all died of tuberculosis, as did No. 3-55 of group IA

TABLE III

Protocol of Experiment on Vaccination of Guinea Pigs with Different Preparations of BCG

Group	No. of animals	BCG injection Mar. 15-16, 1949			Tuberculin test Apr. 26, 1949	Challenge infection May 6, 1949	Survivors sacrificed on
		Strain	History of vaccine	Viable units in dose injected			
IA	16	Standard vaccine	Used within 48 hrs. of preparation of vaccine	13×10^3	0.0005 mg. PPD	13×10^6 bacilli*	7/15/1949
IB	8	" "	" "	"	"	Sterile medium	7/14/1949
IIA	17	Culture in Tween-heated serum filtrate	Grown for 8 days. No further treatment. No storage	12×10^5	"	13×10^6 bacilli	7/18/1949
IIB	8	" "	" "	"	"	Sterile medium	7/14/1949
III	17	" "	Grown for 8 days. Culture stored for 3 wks. at 4°C.	13×10^5	"	13×10^6 bacilli	7/19/1949
IV	"	" "	Grown for 8 days. Culture stored for 6 wks. at 4°C.	35×10^5	"	13×10^6 bacilli	7/19/1949
V	16	Sterile Tween-serum filtrate		0	0.005 mg. PPD	13×10^6 bacilli	7/14/1949

* Culture of human strain "Amerzanga" grown for 8 days in Tween-bovine albumin medium.

which had received the standard vaccine. The other two fatal cases, No. 3-04 of group IA and No. 3-79 of group IV, died 31 and 40 days after challenge inoculation. The only microscopic evidence of generalized tuberculosis in No. 3-04 was enlargement of the axillary lymph nodes, which on histological examination showed tubercles and early caseation. Sections of the other organs

TABLE IV

Response of Guinea Pig to Vaccination with BCG and to Infection with Virulent Human Tubercle Bacilli

No.*	Weight‡	BCG lesions		Necropsy findings						
				Spleen*		Liver		Lungs	Lymph nodes‖	
		Diameter on 3/23	Ulceration on 3/30	Size	Lesions§	Enlargement	Lesions§	Lesions§	Tracheobronchial	Axillary
Guinea pig group IA*										
	gm.	*mm.*		*mm.*						
3-55	Dead 6/30	0	—	65 × 25	Many necrotic	+++	Many necrotic	Many	+++C	C
2-39	500–580	3	—	50 × 22	" large	+	Few large	Few	+++C	"
2-64	550–670	5	—	45 × 21	" "	—	" small	"	+	"
2-72	580–740	4	—	42 × 20	" "	—	" "	Many	+++	"
3-73	430–690	3	—	40 × 16	Few small	—	0	0	+	"
3-05	615–715	5	—	40 × 15	Many large	—	Few small	0	++	
3-03	590–700	3	—	36 × 17	Few "	±	" "	Few	+	"
3-54	330–565	3	—	35 × 20	" "	—	" "	"	++	"
2-47	375–610	3	—	35 × 15	" small	—	" "	" small	++	"
3-14	595–750	5	—	35 × 14	" "	—	0		—	"
2-30	560–685	1	—	33 × 17	" large	—	Few small	"	+++	"
3-56	275–550	3	—	33 × 15	" "	—	" "	"	++	"
2-36	340–605	1	—	32 × 15	0	—	0	0	+	
3-59	330–555	3	—	32 × 13	Few large	—	0	Few	—	"
2-28¶	335–590	2	—	30 × 15	" small	—	Few small	0	+	"
3-04	Dead 6/6	5	—	32 × 13	0	—	0	0	—	"
Guinea pig group IB*										
3-87	295–610	3	—	35 × 12	None	—	None	None		
2-32	570–710	2	—	34 × 16	"	—	"	"		
3-06	540–725	2	—	34 × 11	"	—	"	"		
3-94	330–675	3	—	30 × 13	"	—	"	"		
3-64	325–625	1	—	30 × 13	"	—	"	"		
3-32	440–600	3	—	30 × 12	"	—	"	"		
2-73	255–560	1	—	30 × 11	"	—	"	"		
2-70	600–700	2	—	30 × 10	"	—	"	"		

* Animals arranged in order of decreasing spleen size.

‡ The two figures given under "Weight" refer to the weight of the animal at the beginning of the experiment (just before vaccination with BCG) and at the end.

§ The data presented in this table refer only to macroscopic findings. The expression "few" lesions indicates that less than 10 isolated lesions were seen on macroscopic examination. "Many" lesions indicates more than 10.

‖ C = caseation. Only evidence of macroscopic caseation was recorded.

¶ Non-specific death.

TABLE IV—*Continued*

No.*	Weight‡	BCG lesions		Necropsy findings						
				Spleen*		Liver		Lungs	Lymph nodes‖	
		Diameter on 3/23	Ulceration on 3/30	Size	Lesions§	Enlargement	Lesions§	Lesions§	Tracheobronchial	Axillary
Guinea pig group IIA*										
	gm.	*mm.*		*mm.*						
3-90	375–465	12	+	57 × 27	Semiconfluent	+	Many large	Many	+++	C
3-18	610–565	"	+	45 × 24	"	+	Many small	Few	+	"
2-56	520–560	"	+	45 × 22	Many large	−	Few small	"	−	"
3-08	570–460	"	+	45 × 22	Confluent "	+	" large necrotic	0	+	"
2-35	665–630	"	+	45 × 22	Many "	++	Many small	Many	+++C	
3-68	355–615	"	−	43 × 19	Few "	+	" "	Few	+++	"
3-67	360–570	"	+	40 × 22	" "	−	" "	"	+++	"
3-20	480–700	"	+	37 × 15	0	−	0	"	+	
3-09	600–705	"	+	35 × 15	Few small	−	Few small	0	+	
3-66	325–570	"	+	35 × 15	" "	−	" large necrotic	Few	−	"
3-17	555–680	14	+	34 × 16	0	−	Few	0	±	"
3-10	620–675	10	+	34 × 13	Few small	−	0	0	+	"
3-62	310–580	12	+	34 × 11	" "	−	0	0	±	
3-12	530–595	"	+	33 × 16	Many large	−	Many small	Few	++	"
2-48	260–505	10	+	32 × 13	Few "	±	" "	"	+++	"
3-57	270–525	"	+	32 × 13	" small	−	0	Many	++	"
3-71	320–590	"	+	30 × 15	0	−	Few small	0	+	"
Guinea pig group IIB*										
3-70	360–665	12	+	35 × 15	None	−	None	None		
2-50	455–655	"	+	33 × 15	"	−	"	"		
3-49	580–725	"	+	33 × 15	"	−	"	"		
3-97	320–635	"	+	33 × 13	"	−	"	"		
2-44	330–545	"	+	32 × 14	"	−	"	"		
4-00	330–605	"	+	32 × 12	"	−	"	"		
2-43	550–765	15	+	31 × 14	"	−	"	"		
3-48	580–650	12	+	28 × 13	"	−	"	"		

TABLE IV—*Continued*

No.*	Weight‡	BCG lesions		Necropsy findings							
				Spleen*		Liver		Lungs	Lymph nodes	
		Diameter on 3/23	Ulceration on 3/30	Size	Lesions	Enlargement	Lesions§	Lesions§	Tracheobronchial	Axillary
Guinea pig group III*										
	gm.	*mm.*		*mm.*						
2-52	600–535	14	+	54 × 24	Semiconfluent large	++	Many large necrotic	Many	++++C	C
3-28	585–660	10	+	54 × 22	Many large	−	0	"	+++	"
3-16	680–625	"	−	50 × 25	Semiconfluent large	++	Many small	"	++++C	"
2-26	330 × 560	8	+	45 × 21	Few large	−	Few "	"	++++	"
2-41	595 × 670	10	+	45 × 20	Many "	+	" "	"	++	"
2-49	230 × 465	8	+	45 × 18	Few necrotic	−	" large	Few	+++	"
3-77	300–560	10	+	42 × 20	Many large	−	"	Many	++	
2-42	260–520	6	+	39 × 22	Confluent	−	" necrotic	"	++++	"
3-75	300–480	8	+	37 × 22	Few large	−	Few small	Few	+++C	"
3-46	490–635	10	+	37 × 17	" "	+	Many small	Many	+++	"
3-29	650–675	"	+	35 × 14	Many small	+	" "	Few	++	
2-38	640–670	12	+	35 × 12	" "	−	0	0	+	"
3-22	520–590	14	+	33 × 17	" "	−	0	Few	±	"
3-74	370–625	10	+	33 × 13	" "	−	Few small	0	++	"
3-13	560–625	12	+	30 × 12	" "	−	" "	Few	±	"
3-72	325–565	10	+	27 × 13	" "	−	0	0	±	"
2-31	265–470	"	+	35 × 17	" large	−	Few small	Many	+++C	

TABLE IV—*Continued*

No.*	Weight‡	BCG lesions		Necropsy findings						
				Spleen*		Liver		Lungs	Lymph nodes	
		Diameter on 3/23	Ulceration on 3/30	Size	Lesions	Enlargement	Lesions§	Lesions§	Tracheobronchial	Axillary
Guinea pig group IV*										
	gm.	*mm.*		*mm.*						
3-91	350–405	8	+	74 × 30	Semiconfluent necrotic	++	Many large necrotic	Many	++	
3-39	530–505	10	+	58 × 25	" "	++	" "	"	++++	C
3-92	340–535	8	+	55 × 25	Confluent large	−	Few small	Few	++	"
3-81	285–490	"	+	50 × 25	" "	+	Many "	Many	+++	"
3-85	210–530	"	+	43 × 18	Many large	+	" "	Few	+	"
3-33	575–645	10	+	40 × 15	" "	+	Many small	Many	++++C	"
2-29	595–635	"	+	38 × 18	" "	−	Few small	"	+++	"
3-78	305–605	"	+	37 × 16	" "	−	0	0	+	
2-33	555–650	"	+	35 × 20	" "	−	Few small	Many	++++C	"
2-46	640–790	"	+	35 × 17	Few small	−	0	Few	++	
2-57	555–670	"	+	35 × 15	" "	−	Few small	0	++	"
3-26	675–700	"	+	35 × 15	" large	−	0	Few	++	"
3-52	475–800	"	+	35 × 13	0	−	0	0	±	
3-80	335–640	6	+	33 × 15	Few small	−	0	0	+	"
3-86	270–635	6	+	32 × 14	" "	−	0	Few	+	"
3-31	645–665	10	+	30 × 15	" "	−	Few small	"	+++	"
3-79¶	Dead 6/15	8	+	30 × 12	" "	−	0	0	−	"

TABLE IV—*Concluded*

No.*	Weight‡	Necropsy findings						
		Spleen*		Liver		Lungs	Lymph nodes	
		Size	Lesions	Enlargement	Lesions§	Lesions§	Tracheobronchial	Axillary
Guinea pig group V*								
	gm.	*mm.*						
3-88	Dead 7/13	90 × 28	Many necrotic	++	Semiconfluent necrotic	Many	+++C	C
3-96	" 7/4	75 × 25	Semiconfluent necrotic	+++	Confluent necrotic	"	±	"
3-82	305–590	73 × 34	Confluent necrotic	+++	" "	"	+++	"
3-35	Dead 7/8	55 × 24	Many necrotic	+++	Semiconfluent necrotic	"	++C	"
3-50	" 7/14	53 × 30	Semiconfluent necrotic	+++	" "	"	+++C	"
3-34	550–630	53 × 30	Semiconfluent	++	Many small	"	++++C	"
3-43	530–600	53 × 25	Many small	++	" "	Semiconfluent	+++	"
3-89	Dead 6/9	50 × 27	Semiconfluent necrotic	+++	Semiconfluent necrotic	Many	++C	"
2-61	" 6/27	50 × 24	Many necrotic	+++	Many necrotic	"	+++C	"
3-83	" 6/22	50 × 20	" "	+++	Semiconfluent necrotic	"	+++C	"
2-34	680–750	47 × 25	Semiconfluent necrotic	++	Many necrotic	"	+++	"
2-27	365–550	47 × 24	Many necrotic	++	Many small	"	+++	"
3-36	Dead 6/25	45 × 21	" "	+++	" necrotic	"	+++	"
3-84	" 6/14	45 × 19	" "	+	Few "	Few	+++C	"
3-93	300–610	43 × 23	Semiconfluent necrotic	+++	Many "	Many	+++	"
3-40	Dead 7/14	40 × 20	Few small	+++	Semiconfluent necrotic	Semiconfluent	+++C	"

failed to reveal any abnormality. This animal may have died as a result of injury, for the peritoneal sac contained a considerable quantity of bloody fluid. The axillary and inguinal lymph nodes of No. 3–79 were enlarged and caseous, and there were a few necrotic foci in the spleen but no other macroscopic or microscopic evidence of generalized tuberculosis. The cause of death was not determined.

The results of the various procedures carried out in the course of the experiment can be briefly summarized as follows:—

In all animals which had received the standard BCG vaccine (groups IA and IB) the slight local ulcer observed at the site of vaccination had healed by March 23, 1 week after the injection of the vaccine. At this time there were left only small hard red lumps between 3 and 5 mm. in diameter. These persisted until April 6 and all had completely disappeared by April 20. All animals which had received the fresh BCG culture grown in Tween-serum filtrate medium (groups IIA and IIB) exhibited lesions exceeding 10 mm. in diameter and reaching up to 15 mm. The lesions were in general slightly smaller in animals vaccinated with the culture stored for 3 or 6 weeks (groups III and IV). All animals of groups IIA, IIB, III, and IV (*i.e.* those receiving the Tween cultures fresh or stored) exhibited on March 30 an ulcer which was found to be healed on April 13 (in only one animal of group IIB did the ulcer remain open until April 20). It is clear therefore that the local lesions developing from the intracutaneous injection of BCG were considerably larger, and persisted much longer, in the animals receiving the cultures grown in the medium containing Tween and filtrate of heated serum than in the animals receiving the standard vaccine. It may be inferred from the results of the experiment described earlier in this paper, in which two guinea pigs were inoculated with various doses of subcultures, in the same fluid medium, of the two substrains of BCG that this difference was due principally to the larger number of viable organisms in the Tween-serum filtrate cultures.

All vaccinated animals exhibited intense tuberculin allergy when tested on April 26; while the control animals (group V) failed to react to a 0.005 mg. PPD. The severity of the tuberculin reaction was almost identical in all vaccinated groups, whether it was measured in terms of the average diameter of the erythematous area (varying from 10 × 12 mm. to 20 × 20 mm.), or by the proportion of animals which showed the "*cocarde*" or triple response, consisting of a livid center, an inner white zone, and an outer erythematous area.

A large mass, at first hard but later becoming fluctuant, developed at the site of the challenge infection in all animals. Ulceration of this abscess occurred much earlier in the vaccinated than in the non-vaccinated animals (Table V); amongst the vaccinated guinea pigs those of group IV, which had received the BCG culture stored for 6 weeks in the refrigerator, developed ulcers somewhat later than those of the other groups.

The response of the guinea pigs to inoculation with virulent tubercle bacilli is given in Table IV. The animals within each group are tabulated in order of decreasing spleen size as this was considered the most objective single index of the intensity of the infection. At the conclusion of the experiment all the unvaccinated animals had either died of tuberculosis or showed severe tuber-

culous lesions of the liver, spleen, and lungs. All animals which had received BCG but no challenge infection were gaining weight and in none of them were tuberculous lesions found by macroscopic or microscopic examination. Among the vaccinated animals the only death from tuberculosis occurred in group IA,[4] which had received the standard vaccine, but many other animals of this group appeared to be less severely diseased than animals of the other vaccinated groups. It is obvious that the severity of the disease varied so much from one animal to the other within each group and that there was so much overlapping from one vaccinated group to the other, that the differences observed had no significance. It is of particular interest to point out that no significant difference could be detected between the animals of groups IIA, III, and IV. In each group there were two or three guinea pigs that were losing weight and that had extensive lesions of the spleen and lungs, and moderately severe lesions of the

TABLE V

Date of Ulceration of the Abscess Produced at the Site of Injection (on May 6th) of Virulent Tubercle Bacilli

Group of guinea pigs	No. of animals	No. of animals showing ulceration of subcutaneous abscesses for the first time on the following dates:										
		5/11	5/18	5/25	6/1	6/8	6/15	6/22	6/29	7/6	7/13	No ulceration
IA	16	7	5	1	2	1						
IIA	17	4	10	3	0	0						
III	16	1	9	4	2	0						
IV	17	2	2	6	4	0	2	0	0	0	1	
V	16	0	1	3	6	2	0	1	1	0	0	2

liver. The balance of the animals in each group showed lesions of varied but minor severity, some exhibiting only three or four nodules in a spleen of normal size with no macroscopic lesions in the other internal organs.

DISCUSSION

Some of the findings described in the present paper provide information concerning the lack of pathogenicity of the BCG cultures grown in the medium containing Tween and the soluble fraction of heated human serum. It is true that the intracutaneous injection of 0.05 cc. of culture grown in Tween-serum

[4] It is worth noting that guinea pig 3-55 of group IA which died of tuberculosis had a negligible reaction at the site of injection of BCG (see table IV). Nevertheless this animal reacted strongly to the tuberculin test, the tuberculin reaction measuring 12 × 10 mm. and exhibiting a white center.

filtrate medium resulted in the appearance of local lesions more severe than those produced by 0.05 cc. of standard vaccine. In order to evaluate the significance of this difference however, it must be kept in mind that the numbers of viable bacterial units (determined by plate counts) were much greater in the Tween culture (approximately 10^7 per cc.) than in the standard vaccine (10^5 per cc.). When the two BCG substrains were cultured in the same medium it was found that the intracutaneous injection into normal guinea pigs of doses containing similar numbers of viable units, produced lesions indistinguishable in size and severity. This finding strongly suggests that the bacterial cells which had grown diffusely in Tween-serum filtrate medium had retained the same intrinsic degree of "attenuation" as those present in the vaccine prepared by the standard technique. It illustrates furthermore the fallacy of expressing in terms of units of volume or weight the doses of a living vaccinating agent in the absence of knowledge of its viability or physiological activity.

Although many more living organisms (approximately 100 times as many) were present in the Tween-serum filtrate cultures than in the standard vaccine, and although the former preparations produced larger lesions than the latter at the site of the intracutaneous injection, the intensity of tuberculin sensitization induced was essentially the same in the four groups of animals vaccinated with the four different preparations mentioned in Table III. Here again, experiments carried out with known numbers of viable bacterial units provide a clue for the analysis of these findings. The data presented in Table I reveal that a definite degree of sensitization could be achieved by the injection of doses of BCG culture containing fewer than 100 viable bacterial units. Moreover the intensity of the sensitivity to tuberculin, measured 6 weeks after vaccination, reached an apparent maximum with a vaccinating dose containing 4000 viable units; even an increase many hundredfold in the number of viable units injected for vaccination failed to bring about a detectable increase in the intensity of the sensitivity to tuberculin. It is clear therefore that the design of the experiment outlined in Table III did not permit a comparison of the efficacy of the different preparations used as vaccines, since the numbers of viable units in all these preparations were far greater than the minimal number required for inducing the maximum level of tuberculin allergy detectable by the technique employed.

It is generally accepted that vaccination with BCG results in a definite degree of multiplication of the bacilli *in vivo* and it is probably for this reason that the degree of tuberculin allergy was relatively independent of the vaccinating dose in the experiment under consideration. Freund has established that amounts of heat-killed tubercle bacilli of the order of 0.1 to 0.003 mg. resuspended in mineral oil are required in order to induce an appreciable degree of tuberculin allergy in guinea pigs; the same dose of heat-killed bacilli in aqueous media

would fail to sensitize (6).[5] The amount of bacillary material represented by these weights is many millions of times larger than the weight of living cells of BCG (in aqueous media) which was found adequate to produce maximal sensitization in the present experiment. It appears almost certain, therefore, that the degrees of allergy recorded in the present paper could not be attributed to the mass of bacillary material present in the vaccinating dose but reflect rather an extensive multiplication of the bacilli *in vivo*.

One may assume also that the protection against infection induced in the guinea pigs of groups IA, IIA, III, and IV (Table IV) was the outcome of the multiplication of the vaccinating inoculum *in vivo*. No statistically significant difference could be recognized in the degrees of resistance exhibited by the different groups of vaccinated animals. All had caseous lesions at the site of inoculation (although the ulcer healed rapidly in some of them), and all had enlarged axillary lymph nodes which were usually caseous. In all groups a few guinea pigs exhibited severe tuberculous lesions of the liver, spleen, and lungs while the lesions in most other individuals were confined to a few large nodules in the spleen and small foci in the lungs and liver. Each group also contained some animals in which the only macroscopic lesions of the internal organs were a few foci in the spleen, which itself was not enlarged.

It must be emphasized, however, that differences in the resistance to infection may have existed but could not be brought out under the conditions of the experiment. Indeed, the technique used in the challenge infection test (subcutaneous infection) was not adequate for the measurement and analysis of resistance to infection as it did not lend itself to a clear differentiation between allergy and antibacterial immunity. The allergic reaction elicited by the introduction of the virulent bacilli probably caused a walling off, or even a shedding, of a significant percentage of the infective inoculum thereby decreasing the effective challenge dose. It is possible, indeed likely, that marked differences in the degree of resistance to infection in the four groups of vaccinated animals could have been brought about by changing certain of the possible variables in the experiment:—the amount of vaccine used, the size of the challenge dose, the time elapsed between vaccination and challenge infection, the route of infection, etc.

The experimental results described in this and the preceding paper do not allow therefore a comparison of the antigenic efficacy—either as sensitizing or as immunizing agents—of the four different BCG preparations used for vaccination. They permit one only to state that the culture grown in the Tween-serum filtrate medium compares well qualitatively with the vaccines prepared by the conventional technique and from the quantitative point of view, may even present definite advantages over the latter. The content in viable cells of

[5] Dr. Freund informs us that a limited, but definite degree of tuberculin allergy can be induced in some guinea pigs by amounts of heat-killed bacilli of the order of 0.001 mg. and even one-tenth this amount resuspended in oil.

the Tween-serum filtrate culture is extremely high as shown by colony counts and by the ability of minute amounts of culture to sensitize guinea pigs to tuberculin. Moreover, the number of viable cells is predictable and remains remarkably constant even after prolonged periods of incubation or of storage. This stability should greatly facilitate the planning of vaccination experiments and the analysis of their results.

Other minor advantages of the dispersed cultures deserve perhaps some brief mention, namely the rapidity with which they can be obtained, the fact that they require for their preparation only standard bacteriological techniques, the elimination of the transfer operations and of the necessity of grinding the bacterial growth before distribution of the final product, their stability in the very medium in which they are grown, which allows ample time for the performance of the control tests required of biological products. Nevertheless, it is obvious that the technique of cultivation of BCG described in these reports should be regarded at present only as experimental procedure; it constitutes merely a demonstration that, by adequate cultural methods, one can obtain suspensions of BCG organisms exhibiting stable physiological and antigenic activity.

SUMMARY

Groups of guinea pigs were vaccinated by the intracutaneous route with cultures of BCG grown in a liquid medium containing Tween 80 and the soluble fraction of heated human serum. After the cultures had been stored at 4°C. for various periods of time, the antigenic response was compared with that of another group of guinea pigs receiving standard BCG vaccine prepared by the conventional technique.

The local lesions occurring at the site of injection of cultures in Tween-serum filtrate medium were more severe than those produced by the standard vaccine. It was shown that this difference was probably due to the much larger number of viable bacilli in the former preparations. A marked degree of sensitization could be produced with culture dilutions containing as few as 10 viable units (single bacilli or small clumps). Slightly larger doses of BCG led to the highest degree of tuberculin allergy detectable by the technique employed. Further increases in the dose of vaccine failed to alter the level of sensibility when the animals were tested with tuberculin 5 weeks after vaccination.

The same degree of sensitization was achieved by vaccination with 0.1 cc. of either the standard vaccine or any of the fresh or stored cultures in Tween-serum filtrate medium. It was shown that these doses contained numbers of living bacilli far greater than the minimal number required to induce maximal sensitization.

Under the conditions used, the guinea pigs vaccinated with cultures of BCG (fresh or stored) grown in the Tween-serum filtrate medium exhibited a marked degree of resistance to subcutaneous infection with virulent tubercle bacilli.

The authors wish to extend to Dr. Joseph Aronson, of the Henry Phipps Institute, their appreciation for valuable advice and generous help in different phases of the work reported in this paper.

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