THE MULTIPLICATION OF TUBERCLE BACILLI WITHIN NORMAL PHAGOCYTES IN TISSUE CULTURE

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PLATES 5 TO 7

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Tubercle bacilli can survive for prolonged periods of time within the phagocytic cells of susceptible and resistant hosts (1–3). In sections of autopsy material from human beings and animals dead of tuberculosis, as well as in tissue cultures infected with tubercle bacilli, one always finds phagocytic cells filled with bacilli; this is particularly true of the monocytes and their derivatives (4, 5). For this reason, it has been postulated that tubercle bacilli can multiply within the host cells (6, 7). It is generally agreed that the mononuclear phagocytes harbor a great number of tubercle bacilli at certain stages of the disease (8–10), and that interference with the multiplication and survival of the bacilli within the phagocytes is an important feature of acquired immunity. There is a large amount of experimental evidence in support of this view (11–13).

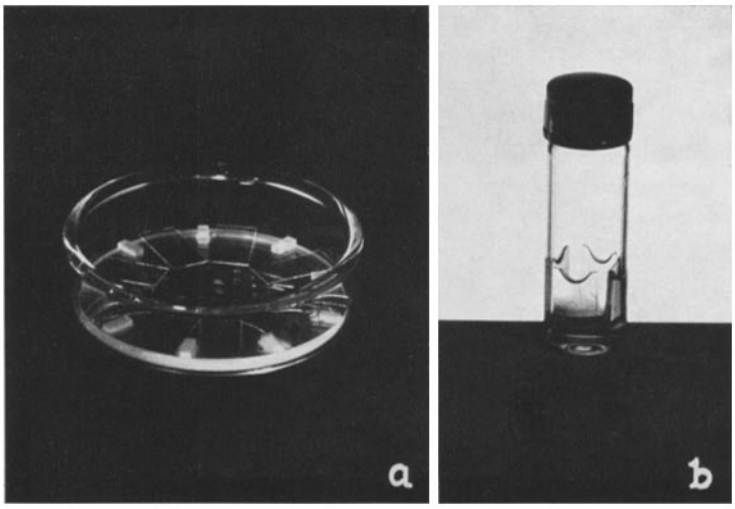
In the present paper a technique will be described which permits the quantitative study *in vitro* of the multiplication of tubercle bacilli within mononuclear phagocytes derived from peritoneal exudates of normal guinea pigs. A later publication will deal with the bearing on acquired immunity of inhibition of bacterial multiplication within phagocytes.

Material and Methods

Bacterial Cultures.—The following strains of tubercle bacilli were used: the virulent human strain H37Rv, the attenuated strains R1Rv, BCG substrain Phipps, and BCG substrain Tice (called BCG III and II in reference 14), and the strain H37Ra, an avirulent variant of H37Rv. All cultures had been maintained for several years in a liquid medium containing 0.05 per cent Tween 80 and 0.5 per cent bovine albumin fraction V (15). In general, 7-day-old cultures were used for the experiments. In order to remove the larger clumps of bacilli, the cultures were centrifuged at a slow speed for about 5 minutes. The optical density of each supernate was measured with the spectrophotometer (Coleman junior at $\lambda = 650$ mμ). By varying the length of time of centrifugation, all supernates were adjusted to an optical density of 0.046 (corresponding to 90 per cent transmittance). Supernates of this density contained approximately 2 million viable units consisting of individual tubercle bacilli or small clumps of bacilli (16). From these standardized bacterial suspensions dilutions were prepared in the original culture medium.

Animals.—Inbred albino guinea pigs of Rockefeller Institute stock were used. Most of them were females weighing approximately 500 gm.

Exudates.—Glycogen was used as a chemotactic agent for the production of peritoneal exudates (17). The animals were injected intraperitoneally with 10 ml. of a solution containing 0.01 mg. of glycogen per ml. in physiological saline. 5 to 7 days later they were bled to death by heart puncture, the blood was allowed to clot, and the serum was collected after it had stood 1 hour at room temperature. After the animals had died, 30 ml. of Hanks' physiological solution (18) containing 1:20,000 heparin was injected into the peritoneal cavity immediately after which the abdomen was kneaded gently in order to promote the suspension of the cells in the fluid injected, then opened, and the fluid withdrawn with a pipette through a sieve tube. About 27 ml. of fluid could thus be recovered from a single animal. A drop of the exudate was immediately examined microscopically with the low power magnification of the microscope and counts were made in a Spencer hemocytometer after diluting the cell suspension in a white cell mixing pipette. In many experiments, the exudates of 2 guinea pigs were mixed.



TEXT-FIG. 1 (*a*). Petri dish with the glass disc and the slides. ×0.73. (*b*) Culture tube with 4 slides and 0.6 ml. of liquid medium. ×0.77.

Preparation of the Phagocyte Culture.—Fresh homologous serum in a final concentration of 2.5 per cent was added to the exudate withdrawn from the peritoneal cavity. Aliquots of 9 ml. exudate were then mixed with 1 ml. of dilutions of the bacterial suspensions standardized as described above. To the control sample 1 ml. of culture medium was added. Without delay each mixture was poured into small Petri dishes (5 cm. diameter). The latter contained a removable glass disc at the periphery of which 8 small pieces of glass had been sealed radially to divide the surface into 9 sectors. A small slide, obtained by cutting a coverslip (No. 1 22 × 22 mm.) in 3 identical strips, was placed in each sector (Text-fig. 1 *a*). The Petri dishes with the exudates were then kept for 1 hour in the incubator at 37°C. to allow the exudate cells to settle on the slides and simultaneously to engulf tubercle bacilli. The phagocytic cells adhered to the surface of the slides, so it was possible to wash the latter in Hanks' solution to free the cells of exudate, as well as of erythrocytes and of tubercle bacilli which had not been phagocytized.

To prevent the phagocytes from floating away during cultivation, the slides with the cells on the upper side were covered with a thin film of formvar (19, 20). This was done by de-

positing a drop of a 0.4 per cent solution of formvar[1] in ethylene dichloride on the surface of chilled physiological saline in a Petri dish. A thin film formed immediately. When the slide was placed beneath this membrane and slowly lifted, the formvar folded around it. The preparation was then washed and placed in a small screw-capped vial (47 × 15 mm.). For tight sealing, the original washers were replaced by rubber. Each tube contained 0.6 ml. of liquid culture medium, and usually 4 slides were placed vertically in it, the side with the cells facing the wall of the tube. The slides were held in position by capillary force within the space between slide and tube (Text-fig. 1 *b*). From each Petri dish with 9 slides 2 culture tubes were prepared, giving 4 duplicate sets.

Tissue Culture Medium.—The culture medium consisted of Hanks' solution, 80 per cent fresh homologous serum, penicillin 50 units per ml., and streptomycin 5 γ per ml. The tubes received 5 per cent CO_2 in air. The cultures were incubated at 37°C. in a wheel rotating at a speed of 4 R.P.M. at an angle of 30° from the vertical. The medium in the culture tubes was changed after the 1st and the 3rd day of cultivation.

Fixation and Staining.—One slide was taken from each Petri dish after the incubation period of 1 hour and from each tube after 1, 3, 5, and 7 days of cultivation. The cells were fixed for 5 minutes in absolute methyl alcohol, washed in water, and the membrane of formvar was removed from the back of the slide. After drying, the preparations were stained with carbolfuchsin at room temperature for 10 minutes (21), washed in water, decolorized by immersion in 3 per cent acid-alcohol for approximately 3 seconds, and counterstained for 10 minutes with Giemsa stain. The slides, washed and dried, were mounted on microscope slides under large coverslips using 50 per cent piccolyte in xylene (General Biological Supply House, Inc., Chicago).

The preparations were then scored according to the following criteria: (*a*) condition of the cells, (*b*) proportion of phagocytes having engulfed tubercle bacilli, and (*c*) number of tubercle bacilli present within each of 100 cells having phagocytized bacilli. These latter counts were made by classifying the cells in groups containing 1 to 2, 3 to 5, 6 to 10, and over 10 stainable tubercle bacilli.

EXPERIMENTAL

Composition of the Exudate.—

The quantity and quality of cellular exudates following injection of a chemotactic agent into the peritoneal cavity of experimental animals depend on the agent used and on the interval of time between injection of the agent and collection of the exudate. Glycogen, known to exert strong positive chemotaxis on migrating cells (17), was chosen because it is a component of normal tissues (22). The results of the following experiment illustrate the chemotactic property of glycogen and the percentage of different types of cells in peritoneal exudates collected at various intervals after injection.

On 7 consecutive days guinea pigs were injected intraperitoneally with 0.1 mg. glycogen in 10 ml. physiological saline. All animals were bled to death on the day following the last injection, and the cells were collected from the peritoneal cavities in 15 ml. of Hanks' solution containing heparin. From each suspension absolute white cell counts were made, a drop was spread on a slide, and 10 ml. of the suspension was poured into a Petri dish in order to allow the phagocytes to settle on slides. Smears and sediments were fixed and stained, and differential counts were made. The results are presented in Table I.

[1] Formvar, grade No. 15–95, polyvinyl formal produced by Shawinigan Products Corp., New York City.

The absolute number of cells recovered from the peritoneal cavity was highest after 1 day, then decreased and remained relatively constant during the following days. As was to be expected, the exudate showed at first a great majority of polymorphonuclear leucocytes, then a prevalence of mononuclear cells. The exudate collected 4 days after injection yielded on the slides chiefly large mononuclear cells, a few eosinophilic cells, and rarely, polymorphonuclear leucocytes. Lymphocytes, apparently, did not settle with the monocytes or were not fixed on the surface of the slide. Mononuclear cells in suspension were found to be actively phagocytic, as also on the slides.

TABLE I

Quantitative and Qualitative Cellular Composition of Peritoneal Exudates of Guinea Pigs at Different Intervals after Injection of Glycogen

Length of time after glycogen	Cells per c.mm.	Differential count						
		Direct smears				Sediments on slide		
		Polymorphonuclear		Mononuclear	Lymphocyte	Polymorphonuclear		Mononuclear*
		Neutrophile	Eosinophile			Neutrophile	Eosinophile	
days								
1	10,400	157	1	36	6	133	3	64
2	3,500	46	0	128	26	11	0	189
3	1,725	2	6	174	18	1	3	196
4	1,800	0	7	166	27	1	1	198
5	2,725	0	3	169	28	0	3	197
6	1,925	0	10	173	17	0	10	190
7	1,925	0	10	169	21	0	9	191

* Mononuclear cells and lymphocytes could not be differentiated. The figures in this column comprise small and large mononuclear cells.

After 1 hour's sedimentation in the incubator the cells on the slides were found to be thickly scattered singly, not piled on one another. The mononuclear cells, which are usually round in smears made from suspensions or blood, showed an irregular outline and long pseudopod-like processes characteristic of migrating cells (Figs. 1 and 2).

Observations on Mononuclear Phagocytes.—

During the period of cultivation on the slides the phagocytes underwent a number of changes. At first they had the characteristics of wandering cells, but after 24 hours of cultivation they appeared rounded with a larger body of cytoplasm. Some had engulfed fragments of dead cells. This process of enlargement continued and the cells often formed groups of 20 or more (Figs. 1 to 4). From the 5th day on the culture consisted mainly of large elongated cells, free

or grouped, each with an oval nucleus. Frequently giant cells appeared consisting of a cytoplasmic mass containing a great number of nuclei. The nuclei were situated on the periphery in some giant cells and were scattered throughout the cytoplasm in others. The cells had now multiplied to such an extent as to cover the slide with a compact sheet of tissue. Some small round cells were still present (Figs. 5 to 7). Thus the cells which had appeared originally as individual monocytes had taken the appearance of macrophages (Figs. 1 and 5). It seems likely that the morphology of the cells was influenced by the structure of the surface of the film of formvar; it differed from one slide to another and also on the same slide. In some preparations the majority of the cells consisted of macrophages in certain areas whereas round cells predominated in others.

In general, the transformation of monocytes into macrophages coincided with the beginning of proliferation of the culture. In other words, mitotic division was observed chiefly in macrophages but rarely in round cells.

Infection of the Mononuclear Phagocytes.—

In order to determine the rate of intracellular multiplication of the tubercle bacilli by counting them within the phagocytes, it was necessary for the infection of the phagocytes to be as uniform as possible throughout the culture. Furthermore, it was of advantage that the majority of the phagocytes should have engulfed no more than 1 or 2 bacilli when the first counts were made.

The best results were obtained by mixing the exudates with diluted bacterial cultures from which the larger clumps of bacilli had been removed by centrifugation. The following experiment demonstrates that the intensity of phagocytosis depends on the properties of the bacterial suspension. Four equal amounts of exudate were infected with either one of the following preparations of a BCG culture: (*a*) undiluted culture, (*b*) the standardized supernate, (*c*) and (*d*) 10-fold and 100-fold dilutions of the supernate. The degree of phagocytosis and the number of tubercle bacilli within the cells were determined on preparations obtained after 1 hour of sedimentation and phagocytosis. Table II represents the results of such an experiment.

It can be seen that the proportion of phagocytes containing tubercle bacilli was largest with the original culture and only slightly smaller with the supernate. Dilution of the supernate resulted in a proportional decrease in the degree of phagocytosis. However, the numbers of bacilli taken up by the individual phagocytes were much smaller in experiments with the supernate than with the original culture. The numbers were not reduced much further by diluting the supernate. Unless otherwise stated, 9 ml. samples of the exudate suspension were infected with 1 ml. of a 10-fold diluted standardized supernate of a centrifuged culture in all subsequent experiments.

Multiplication of Tubercle Bacilli within Phagocytes.—

As shown in preceding sections, mononuclear cells could be cultivated in a thin layer on slides, and it was possible to establish a uniform and minimal intracellular infection of these cells with tubercle bacilli. When infected cultures were studied over a period of 7 days, two types of alterations were observed: the number of bacilli within the monocytes increased steadily; and the cells themselves were affected by the bacilli. The results to be described have led to the conclusion that the increase in number of tubercle bacilli was probably the result of intracellular multiplication of the bacilli.

TABLE II

Relation between the Dilution of the Bacillary Suspension and the Distribution of Tubercle Bacilli (BCG) in Mononuclear Exudate Cells from Guinea Pigs

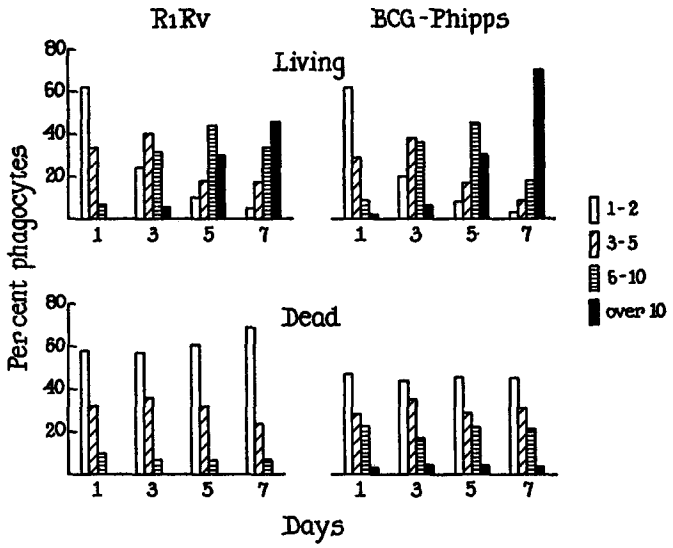
	Suspension as such	Supernate		
		Undiluted	Diluted	
			1:10	1:100
Phagocytosis, *per cent*.....	91.5	75	8.5	1.5
A. 1–2 bacilli	15*	55	71	‡
B. 3–5 "	35	31	21	
C. 6–10 "	35	14	8	
D. >10 "	15	0	0	

* 100 phagocytes containing tubercle bacilli were classified according to the number of stainable bacilli found within them. The figures represent the percentage of phagocytes containing the following number of tubercle bacilli: 1 to 2 (A), 3 to 5 (B), 6 to 10 (C), and more than 10 (D).

‡ No cells were found containing more than 2 or 3 bacilli.

(*a*) *Cultures Infected with Living and Dead Tubercle Bacilli.*—The cultures (R1Rv and BCG-Phipps) were centrifuged to remove large clumps of bacilli. The supernates consisting of dispersed bacillary suspensions were divided into 2 parts, one of which was heated at 56°C. for 1 hour, whereas the other was kept at room temperature. Heating at 56°C. for 1 hour was sufficient to kill all tubercle bacilli as shown by the fact that no growth was obtained by plating the heated samples on culture medium. Heated and unheated suspensions were diluted 10 times and used for infecting the monocyte suspensions. The results obtained with these tissue cultures are given in Table III and Text-fig. 2.

During incubation the number of tubercle bacilli per phagocyte increased in the cultures infected with living bacilli. The majority of cells contained only 1 or 2 bacilli on the 1st day and from 10 to 50 on the 7th day. By contrast, the numbers of bacilli per cell did not change in the cultures of phagocytes to which dead bacilli had been added.



TEXT-FIG. 2. Mononuclear cells infected with suspensions of living and dead tubercle bacilli. The columns represent the number of phagocytes containing 1 to 2, 3 to 5, 6 to 10, or over 10 tubercle bacilli.

TABLE III

Numbers of Stainable Bacilli within Mononuclear Cells in Tissue Cultures Infected with Living and Dead Tubercle Bacilli

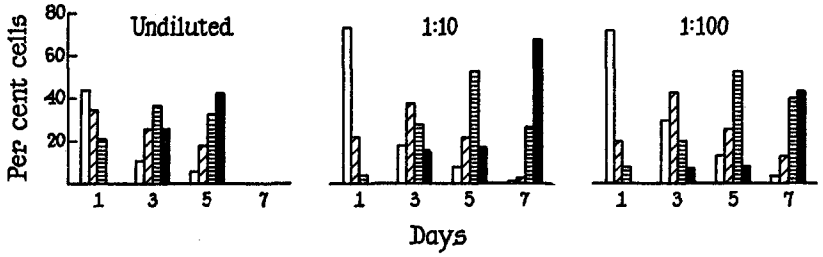
Bacilli	Period of cultivation, *days*..	R1Rv								BCG (Phipps)							
		1		3		5		7		1		3		5		7	
		a	b*	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Living	A. 1–2 bacilli	58‡	61	21	24	9	10	8	5	61	56	20	28	8	6	3	3
	B. 3–5 "	32	33	35	40	18	17	14	17	29	29	38	36	17	9	9	7
	C. 6–10 "	9	6	37	31	46	44	35	33	8	14	36	32	45	31	18	27
	D. >10 "	1	0	7	5	27	29	43	45	2	1	6	4	30	54	70	63
Dead	A. 1–2 bacilli	51	58	58	57	57	61	78	69	50	47	50	44	53	46	47	45
	B. 3–5 "	36	32	29	36	33	32	16	24	34	28	34	35	30	28	36	31
	C. 6–10 "	11	10	12	7	10	7	6	7	16	22	15	17	12	22	15	21
	D. >10 "	2	0	1	0	0	0	0	0	0	3	1	4	5	4	2	3

* Columns a and b represent duplicate counts made on separate slides from two different culture tubes.

‡ See Table II.

(*b*) *Cultures Infected with Various Dilutions of Tubercle Bacilli.*—The increase in the number of tubercle bacilli within infected phagocytes cultivated *in vitro* might be due to mechanisms other than intracellular multiplication. For

instance, it might result from the phagocytosis of bacilli released from cells which had died during cultivation. If this were the case, no increase in numbers of bacilli should be observed in cultures in which only a small proportion of the cells was infected, since bacilli liberated by a dead cell would probably be taken up by phagocytes still free of bacilli.



TEXT-FIG. 3. Mononuclear cells infected with varying dilutions of suspensions of tubercle bacilli (BCG-Phipps). See legend for Text-fig. 2.

TABLE IV

Influence of the Size of the Infective Inoculum on Intracellular Multiplication of Tubercle Bacilli within Mononuclear Cells in Vitro

Dilution of inoculum	Undiluted								1:10								1:100							
Period of cultivation, *days*	1		3		5		7		1		3		5		7		1		3		5		7	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
A. 1–2 bacilli	44*	44	11	11	6	6	—‡		74	66	18	20	8	5	2	2	72	—§	30	22	13	13	4	7
B. 3–5 "	35	36	26	24	18	18	—		22	23	38	40	22	15	3	7	20	—	43	46	26	21	13	12
C. 6–10 "	21	17	37	31	33	38	—		4	10	28	28	53	51	27	27	8	—	20	25	53	56	40	34
D. >10 "	0	3	26	34	43	38	—		0	1	16	12	17	29	68	64	0	—	7	7	8	10	43	47
Phagocytosis, *per cent*	73	74	75	82	75	77	—		7	9	17	16	13	18	15	13	2	1	2	1	2	3	6	1

* See Table II.

‡ No count. Most of the phagocytes were destroyed by the large number of bacilli.

§ No count.

A 7-day-old culture of BCG-Phipps was centrifuged to obtain a supernate of the standardized optical density. One ml. samples of the undiluted bacillary suspension, of 10-fold and 100-fold dilutions of it, were each added to 9 ml. of suspension of exudate phagocytes; the mononuclear cells were then cultivated by the usual technique. The numbers of tubercle bacilli within the phagocytes and the percentage of phagocytosis in these cultures are presented in Table IV and Text-fig. 3.

It is apparent that the bacilli increased in all infected cultures regardless of the dilution of the bacillary inoculum, although multiplication appeared somewhat slower with the diluted cultures. The percentage of cells which were infected during cultivation remained approximately the same. It is noteworthy that infection with the undiluted bacillary suspension resulted in almost complete destruction of the tissue culture after the 7th day.

(*c*) *Cultures Infected with Tubercle Bacilli of Various Degrees of Virulence.*— Whereas virulent and also attenuated strains of tubercle bacilli multiply abundantly in the tissues of normal susceptible hosts (4, 23), the avirulent strain H37Ra is unable to proliferate *in vivo*. It was hoped that the study of the comparative extent of multiplication of virulent, attenuated, and aviru-

TABLE V

Intracellular Multiplication of Different Strains of Tubercle Bacilli within Mononuclear Cells in Vitro

Period of cultivation, *days*....	H37Rv								R1Rv								BCG-Phipps								BCG-Tice								H37Ra							
	1		3		5		7		1		3		5		7		1		3		5		7		1		3		5		7		1		3		5		7	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
A. 1–2 bacilli	52*	53	22	27	12	8	‡	‡	56	63	18	10	4	2	5	‡	47	54	17	16	6	8	1	5	58	47	21	14	7	10	0	1	23	18	21	15	24	19	25	18
B. 3–5 bacilli	30	30	46	50	23	16	—	—	29	28	46	41	9	11	13	—	34	26	48	51	18	18	9	7	27	28	50	50	23	17	12	6	32	29	28	32	34	34	32	33
C. 6–10 bacilli	16	14	23	17	42	44	—	—	13	7	28	28	37	34	27	—	15	16	28	25	37	40	19	25	13	21	25	23	49	47	34	27	28	32	27	32	28	28	27	28
D. >10 bacilli	2	3	9	6	23	32	—	—	2	2	8	21	50	53	55	—	4	4	7	8	39	34	71	63	2	4	4	13	21	26	54	66	17	21	24	21	14	19	16	21

* See Table II.

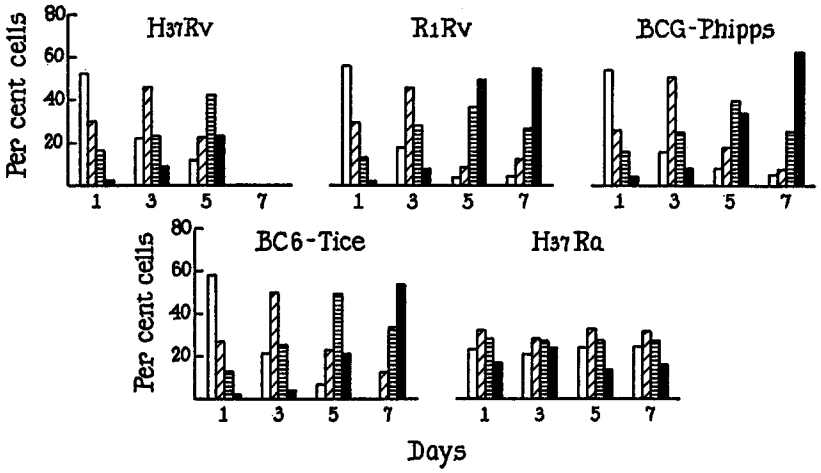
‡ No counts, because the cultures were completely destroyed by the tubercle bacilli.

lent tubercle bacilli within phagocytes cultivated *in vitro* would provide information concerning the validity of tissue culture technique for the study of host-parasite relationship.

From a mixture of two exudates, equal volumes of a phagocyte suspension were infected with diluted standardized suspensions of each of the following strains of tubercle bacilli: H37Rv (virulent), R1Rv, BCG-Phipps, BCG-Tice (attenuated), and H37Ra (avirulent). The tissue cultures were prepared by the method described earlier in this report, and samples were removed after 1, 3, 5, and 7 days of cultivation. The results of this experiment are presented in Table V and Text-fig. 4.

The data show that attenuated as well as virulent tubercle bacilli increased in number within the phagocytes at approximately the same rate. (See Figs. 8 to 11.) By contrast, the bacillary content of the cells infected with the avirulent strain H37Ra did not change.

In cultures infected with the virulent strain, H37Rv, the phagocytes containing large numbers of tubercle bacilli became pycnotic after the 5th day and eventually disintegrated so that only a few macrophages still appeared



TEXT-FIG. 4. Mononuclear cells infected with comparable dilutions of cultures of tubercle bacilli of different virulence: H37Rv (virulent), R1Rv, BCG-Phipps, BCG-Tice (attenuated), and H37Ra (avirulent). See legend for Text-fig. 2.

TABLE VI

Influence of Streptomycin on Intracellular Multiplication of Tubercle Bacilli (BCG-Phipps) within Mononuclear Cells in Vitro

Streptomycin, γ per ml.....	0								5								80							
Period of cultivation, *days*.....	1		3		5		7		1		3		5		7		1		3		5		7	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
A. 1–2 bacilli	55*	42	15	15	6	6	5	5	52	43	15	19	5	11	7	7	53	51	54	44	58	50	55	58
B. 3–5 "	34	36	53	56	16	20	11	12	34	34	57	49	18	19	12	14	31	30	28	24	29	37	32	26
C. 6–10 "	9	20	27	25	59	60	29	26	13	15	23	23	59	62	27	30	16	12	15	22	13	13	10	14
D. >10 "	2	2	5	4	17	14	55	57	1	8	5	9	18	8	54	49	0	7	3	10	0	0	3	2

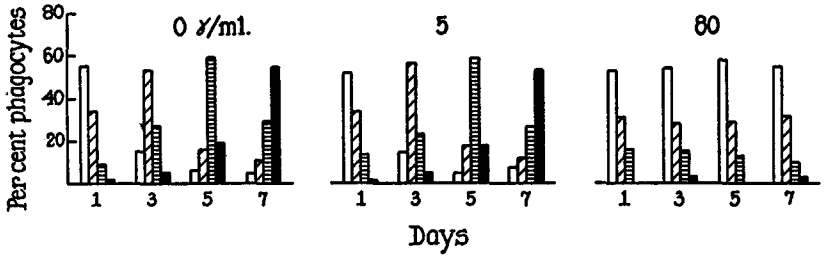
* See Table II.

normal in the culture on the 7th day. This destructive effect of the bacilli was less intensive with the culture R1Rv and still less with BCG-Phipps. Infection with BCG-Tice did not result in any obvious cellular damage (Figs. 11 and 12).

The difference among the strains with reference to their damaging effect upon the phagocytes appeared quantitative rather than qualitative. For instance, monocyte cultures infected with a heavy suspension of BCG-Phipps

were almost completely destroyed by the large number of bacilli which they contained (see Table V), whereas cultures infected with smaller numbers of bacilli remained apparently normal.

(*d*) *Influence of Streptomycin on Intracellular Multiplication.*—In all the experiments described so far, streptomycin was present in the medium in a concentration of 5 γ per ml. This concentration was selected after it had been found in preliminary experiments that it could completely prevent extracellular proliferation of the bacilli without preventing increase in their number within the cells. Much higher concentrations of streptomycin in the culture medium were required to prevent intracellular multiplication. This effect is demonstrated in Table VI and Text-fig. 5.



TEXT-FIG. 5. Effect of streptomycin (γ per ml.) on intracellular multiplication of tubercle bacilli (BCG-Phipps) in mononuclear cells *in vitro*. See legend for Text-fig. 2.

DISCUSSION

In earlier studies of the effect of infection of tissue cultures with tubercle bacilli emphasis has been placed on attempts to bring about the formation of tubercles *in vitro* (6, 24, 26). It has been observed repeatedly that tubercle bacilli multiply abundantly in tissue cultures and that the phagocytic cells become loaded with them. Under the conditions used in the past, however, the bacilli also multiplied so abundantly in the extracellular phase that the tissue cultures finally became overgrown by them (7, 25, 26). In natural or experimental infections *in vivo* large numbers of phagocytic cells are constantly available to clear the tissues of free bacilli. As a result, certain phases of tuberculous disease can be considered as an intracellular infection of the phagocytic system, although necrosis and caseation can create conditions under which extracellular proliferation prevails. Streptomycin has been found to be a useful agent for the study of intracellular multiplication *in vitro* since it is possible with it to suppress extracellular proliferation in concentrations which do not inhibit intracellular multiplication of the bacilli.

In the present study, the phagocytes were deposited on the slides as a single cell layer, thus permitting microscopic observation of the culture with the oil immersion lens. Under the conditions of cultivation, the mononuclear phago-

cytes changed considerably in form and size after a few days; the cells, cytoplasm as well as nuclei, became larger and were stained less intensely than at the beginning of the cultivation. Frequently, giant cells were seen even in uninfected cultures. Similar observations have been made using hanging drop cultures of normal spleen derived from chick embryos (27). The transformation of monocytes into macrophages has been observed repeatedly in tissue culture with spleen (26) or blood (28–30). By the use of the rabbit ear chamber, it has also been found that monocytes migrating from the vessels into the surrounding tissues could later be traced as histiocytes (30). Macrophages or histiocytes can be considered as a functional cell type into which other differentiated cells can transform (31).

The purpose of the present study was to follow the fate of tubercle bacilli within the phagocytes of normal guinea pigs during cultivation *in vitro*. It was observed that the number of bacilli within the cells increased steadily during the period of cultivation, a majority of phagocytes which originally contained only 1 to 2 bacilli becoming eventually loaded with them. The following evidence supports the view that this increase in bacillary content was the result of intracellular multiplication: (*a*) the numbers of bacilli increased in cultures infected with virulent and attenuated strains but not in those infected with the avirulent strain H37Ra; (*b*) the number of bacilli remained unaltered in cultures to which heat-killed bacilli had been added; (*c*) the rate of increase was approximately the same regardless of the proportion of phagocytes infected.

Tubercle bacilli multiplying within the phagocytes caused damage to the host cells; but whereas many of the phagocytes infected with virulent bacilli were destroyed, considerably fewer cells were damaged in cultures infected with attenuated strains. This ability of some strains to destroy the host cells may be of significance in determining the fate of the infection *in vivo*, since destruction of the phagocytic cells may facilitate the spread of the infection. It is noteworthy that the difference among strains was found to be quantitative rather than qualitative. There have been many reports that tubercle bacilli can damage the cells of the host, an effect that may result either in destruction of the phagocytes (25) or inhibition of some vital functions (32–34). Thus two properties at least may be necessary to enable tubercle bacilli to establish a progressive infection,—ability to multiply within the phagocytes and capacity to damage the host cell. Quantitative differences in the latter property may account for the differences in virulence among strains (14).

The concentrations of streptomycin required to prevent intracellular multiplication were found to be much higher than those known to be sufficient for bacteriostasis in cell-free cultures. These findings are of interest in view of the common experience in clinical and experimental tuberculosis that streptomycin often fails to bring about complete elimination of the bacilli.

SUMMARY

A technique has been described for the cultivation *in vitro* of normal mononuclear cells on glass slides in a liquid medium. Under these conditions the monocytes transformed into macrophages which proliferated as in ordinary tissue culture.

These cultures of monocytes could be infected with tubercle bacilli. The numbers of stainable tubercle bacilli within the monocytes increased steadily in cultures infected with virulent or attenuated strains. Evidence is given to support the view that this increase in numbers of bacilli was due to intracellular multiplication. There was no evidence of intracellular bacillary multiplication in cultures infected with an avirulent strain.

Tubercle bacilli multiplying within phagocytes *in vitro* exert a damaging effect upon the host cells. The damage was most obvious in cells infected with a virulent strain.

Tubercle bacilli within phagocytes were protected against the bacteriostatic effect of streptomycin added in a concentration of 5 γ per ml. of culture medium. This permitted the use of streptomycin in infected cultures to prevent extracellular multiplication of the bacilli.

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EXPLANATION OF PLATES

The photomicrographs were made by Mr. Julian A. Carlile.

PLATES 5 AND 6

FIGS. 1 to 7. Tissue culture of monocytes derived from peritoneal exudates in normal guinea pigs 5 days after the injection of glycogen. The monocytes were cultivated on slides covered with a film of formvar in a liquid medium.

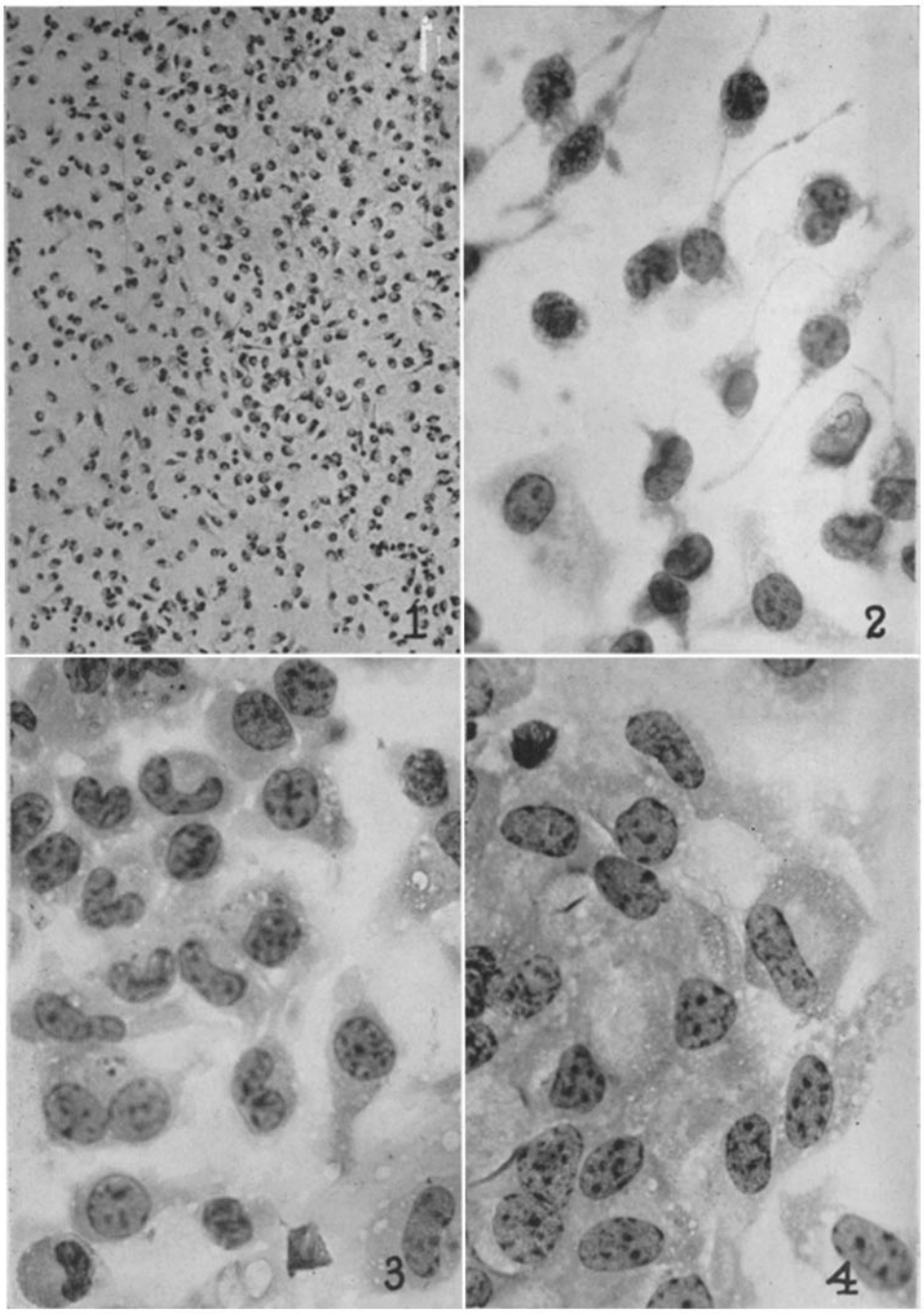
PLATE 5

FIG. 1. Monocytes immediately after they had settled on the slide from the exudate suspension during 1 hour at 37°C. Actively wandering cells in a single layer. × 101.

FIG. 2. Detail of Fig. 1. Most of the cells show long pseudopodia, and only a few phagocytes appear as round cells. × 743.

FIG. 3. Culture after 24 hours. The cells show a larger cytoplasmic body with irregular outlines. In the lower left-hand corner an eosinophilic leucocyte is shown which has still the rounded form. × 743.

FIG. 4. Culture after 3 days. The phagocytes have formed groups. In some instances it is difficult to distinguish them individually. × 743.



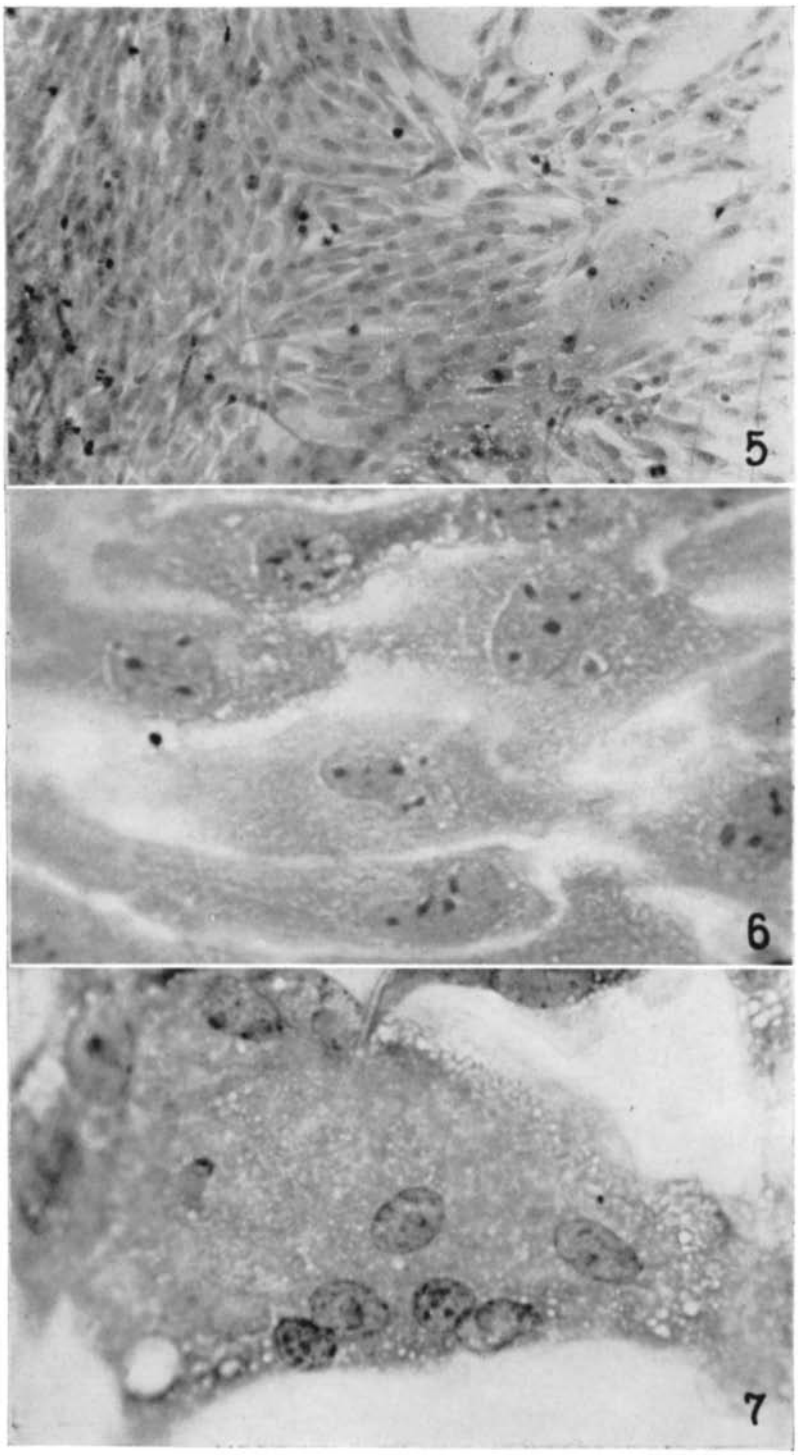
(Suter: Tubercle bacilli in cultured phagocytes)

PLATE 6

FIG. 5. Culture after 5 days. The cells form a tissue-like population covering the slide as an adherent sheet. Cells in mitosis indicate proliferation. The monocytes have become a macrophage culture. × 101.

FIG. 6. Detail of a macrophage culture after 5 days. The enlargement of the cells can be recognized by comparing them with the cells in Fig. 2. × 743.

FIG. 7. Giant cell in a culture after 7 days. Many nuclei are scattered irregularly in a large cytoplasmic body probably as a result of fusion of single cells. × 743.



(Suter: Tubercle bacilli in cultured phagocytes)

PLATE 7

FIGS 8. to 11. Monocyte cultures at different times after infection with an attenuated strain of tubercle bacilli (BCG-Phipps). × 743.

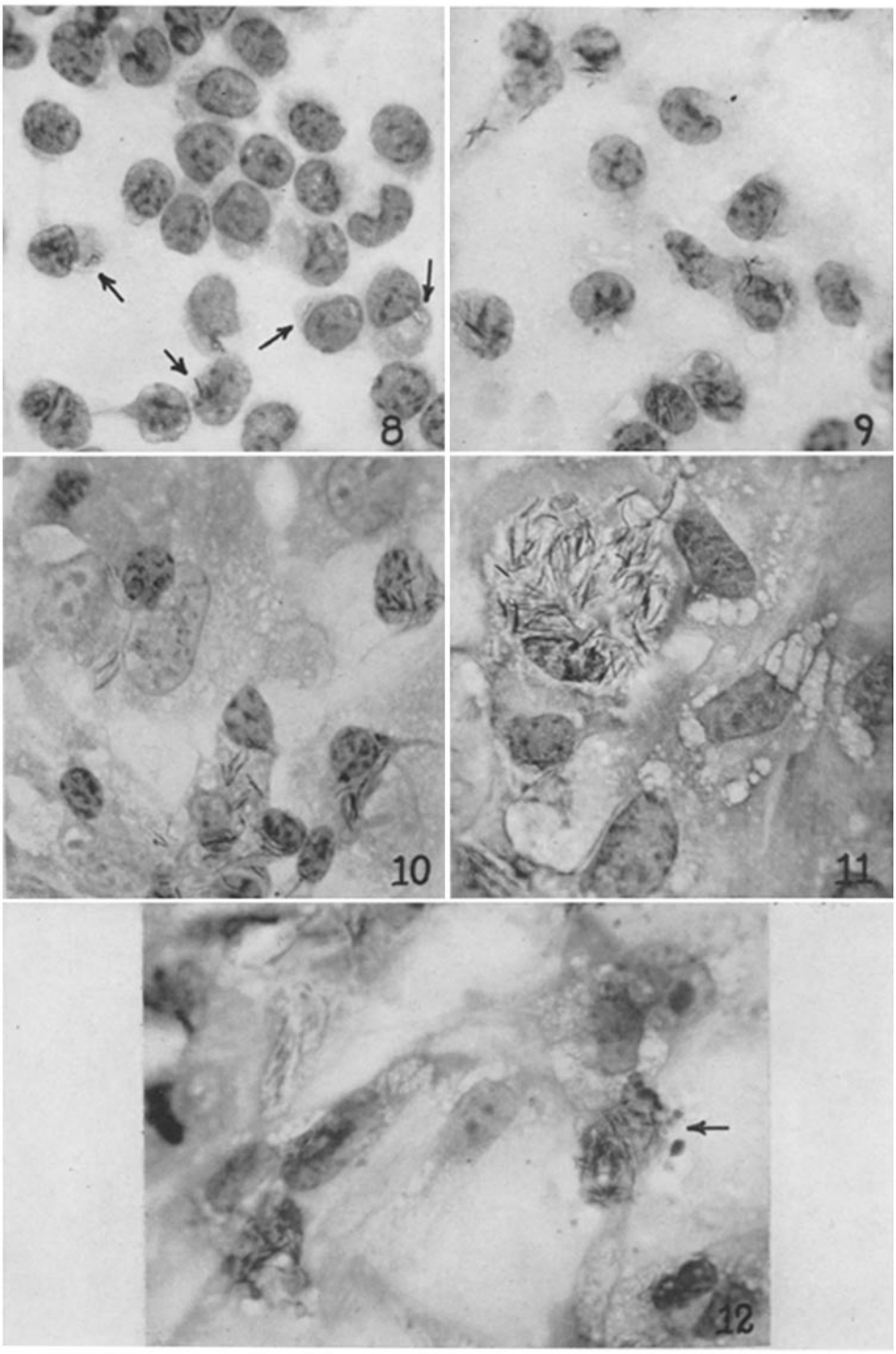
FIG. 8. After 1 day: 4 cells are shown which contain 1 or 2 bacilli (arrows).

FIG. 9. After 3 days: the number of stainable bacilli inside the phagocytes has increased.

FIG. 10. After 5 days: further increase in the number of bacilli. Many cells have transformed into macrophages. The nuclei of those cells which contain tubercle bacilli stain darker than the nuclei of cells without bacilli.

FIG. 11. After 7 days: a cell filled with tubercle bacilli is shown. The nucleus is in a peripheral position.

FIG. 12. Culture infected with virulent tubercle bacilli (H37Rv) after 7 days. A cell is shown with pycnotic nucleus in the process of disintegration (arrow). The non-infected macrophages seem to be unharmed. × 743.



(Suter: Tubercle bacilli in cultured phagocytes)