THE HOST RESPONSE TO CALMETTE-GUÉRIN BACILLUS INFECTION IN MICE*

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BCG (Calmette-Guérin bacillus) is presumed to protect against tuberculosis by virtue of the specific immune response it provokes in the vaccinated subject, but the measurement of this response is beset with technical problems. First, the tubercle bacillus, against which specific resistance must ultimately be measured, is a poor indicator of the resistance generated in response to immunization. Not only does it grow relatively slowly in the tissues of the nonimmune host, but it is also discouragingly resistant to inactivation, even at the height of the immunity generated during infection. Second, the challenge infection itself provokes an immune response which may be prompt and large enough to overshadow most of the effects of prior immunization. As a result, the observed differences between nonimmune and immunized animals are often small, regardless of whether they are estimated by bacterial enumeration or mortality data (1).

These problems can be largely circumvented by using organisms other than the tubercle bacillus to measure the progressive changes in host resistance produced by vaccination with BCG. This approach is made feasible by the very high order of cross-resistance which develops between infections caused by intracellular bacterial parasites. This phenomenon of cross-resistance is dependent upon the well documented observation that when the mononuclear phagocytes of the infected host become highly resistant to infection their activities are not exclusively directed against the infective agent which induced the cellular change (2).

In the present studies, *Listeria monocytogenes* and *Salmonella typhimurium* have been used to measure the development of host resistance after primary BCG infection in the mouse and to study the effect of reinfection with BCG.

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The latter investigation was undertaken in the belief that the benefits of immunization may rest less upon the level of resistance present at the time of reinfection than upon the capacity of the vaccinated animals to produce an anamnestic response.

Materials and Methods

Animals.—Female mice from a specific pathogen-free (SPF) colony maintained at the Trudeau Institute were used at 6–8 weeks of age. This colony was derived from Caesarian-obtained, barrier-sustained progenitors (COBS) supplied by Charles River Breeding Laboratories, North Wilmington, Mass. They have been maintained in strict isolation in an infection-free environment.

Organisms.—The strain of *Listeria monocytogenes* was virulent for SPF mice (intravenous $LD_{50} = 3 \times 10^4$) in which virulence was maintained by continuous passage. Organisms recovered from the spleen were used at the first subculture in tryptic soy broth (Difco Laboratories Inc., Detroit, Mich.). Viable counts were performed on tryptic soy agar (Difco). The strain of *Salmonella typhimurium* was also virulent for SPF mice (intravenous $LD_{50} = 2 \times 10^3$). It was grown in tryptic soy broth and viable counts were made on nutrient agar plates (Difco). *BCG (Rosenthal)* and *Mycobacterium smegmatis* were obtained from the culture bank at the Trudeau Institute, where stock cultures were maintained on an inspissated egg medium (3). Mice were infected with BCG using a 6-day growth from the second or third subculture in Tween-albumin liquid medium (Difco). The BCG cultures were dispersed by ultrasound (Bronwill Biosonik) and diluted in 0.01% bovine serum albumin (BSA) in 0.85% sodium chloride prior to injection. Plate counts were performed on Middlebrook 7H-10 agar (Difco). *M. smegmatis* was used as a 3-day subculture in Tween-albumin liquid medium, and viable counts were made on nutrient agar.

Serum.—Antiserum to *S. typhimurium* was obtained from mice which had been immunized with a heat-killed vaccine. Three doses of 10^9 bacterial cells were given at weekly intervals, the first and third by intravenous injection and the other intraperitoneally. The mice were bled 1 wk after the last injection. The serum was separated, sterilized by membrane filtration, stored at −20°C in small volumes, and used immediately after thawing.

Bacterial Enumeration in Liver, Spleen and Blood.—Organs removed aseptically from individual mice were ground separately with a motor driven teflon-glass homogenizer in a final volume of 10 ml of 0.01% BSA in saline. After serial dilution in the same solution, aliquots were inoculated on suitable agar plates. The number of bacteria in circulation was estimated by plating approximately 0.1 ml of blood from the retro-orbital venous plexus on tryptic soy agar, and multiplying the colony count by 20, on the assumption that the blood volume of a mouse was approximately 2 ml.

Bactericidal Activity of Peritoneal Cells.—A method was devised which employed *M. smegmatis* as a stable viable marker which is readily phagocytosed by peritoneal macrophages and survives well within them (4).

An 18 hr culture of *S. typhimurium* was exposed to ultrasound for 10 sec and the bacteria were counted in a Petroff-Hausser chamber. The culture was then diluted in balanced saline solution (BSS) to a concentration of 5×10^6 bacilli/ml, and added to specific antiserum in the ratio of 4 to 1. This suspension was left at 4°C for 30 min to ensure opsonization. Meanwhile, aggregates were removed from a Tween-albumin culture of *M. smegmatis* by centrifugation before and after exposure to ultrasound for 10 sec. After direct counting and dilution in BSS to a concentration of 5×10^5 bacteria/ml, this suspension was mixed with an equal volume of a 1:10 dilution of the suspension of opsonized *S. typhimurium* making a final suspension containing approximately equal numbers of the two bacterial species. Mice were

injected intraperitoneally with approximately 5×10^4 mixed bacteria in a volume of 0.1 ml. Exactly 15 min later, the mice were killed and their peritoneal cavities were washed out with 2 ml distilled water. Aliquots of appropriate dilutions of the original inoculum and the peritoneal washings were plated in duplicate. Colonies of *S. typhimurium* and *M. smegmatis* were counted after 24 and 72 hr incubation at 37°C, respectively. The two bacterial species were easily distinguishable by means of their different growth rates and colonial morphology. The results were expressed as the "corrected percentage survival" of *S. typhimurium*, which was calculated as follows:

$$100 \times \frac{(\text{Viable count } S.\ typhimurium/\text{viable count } M.\ smegmatis) \text{ recovered}}{(\text{Viable count } S.\ typhimurium/\text{viable count } M.\ smegmatis) \text{ injected}}$$

The use of the above ratio ensured that the estimated survival of *S. typhimurium* was unaffected by such experimental variables as faulty inoculation, transport of organisms from the peritoneal cavity, or incomplete recovery of the inoculum from the peritoneal cavity.

The method described is dependent on two assumptions, namely: (*a*) The bacterial inoculum is rapidly phagocytized after its introduction into the peritoneal cavity. In a preliminary experiment it was found that the mean percentage phagocytosis at 15 min was 99.9% for *S. typhimurium* and 96.3% for *M. smegmatis*. (*b*) No appreciable killing of *M. smegmatis* occurred during the period of observation. Preliminary tests showed that no measurable reduction occurred over a 4 hr period in the number of viable *M. smegmatis* present in the livers or spleens of mice inoculated intravenously.

These results are consistent with those of Cohn (4) who found that *M. smegmatis* was rapidly phagocytized by peritoneal cells, yet survived well within them.

Mouse Macrophage Cultures and Electronmicroscopy.—Mouse peritoneal macrophages were obtained in suspension by washing out the peritoneum with Hanks' BSS containing 10% fetal calf serum (FCS) and 2 units/ml heparin. Aliquots were placed in simple well chambers made from a glass ring fixed to a coverglass. They were incubated at 37°C in an atmosphere containing 5% CO_2. After appropriate intervals, cultures were fixed for 10 min with 1.5% glutaraldehyde in 0.67 M cacodylate buffer (pH 7.4) and 1% sucrose. They were examined by phase microscopy. Peritoneal cells were also fixed in suspension with the same glutaraldehyde fixative and postfixed with 1% osmium tetroxide in 0.05 M acetate-veronal buffer and 5% sucrose at pH 7.4. They were then embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate for electron microscopic examination.

Delayed Hypersensitivity in the Footpad.—2 μg PPD (purified protein derivative) (Parke, Davis and Co., Detroit, Mich.) was injected in a volume of 40 μl diluent (Parke, Davis) into the right hind footpad. After 24 hr, the thickness of each hind foot was measured to 0.05 mm with dial gauge calipers (Schnelltaster, H. C. Kröplin, GmbH, Schluchtern, Hessen, Germany). Since it has been shown that the right and left hind feet of the mouse do not differ in size (5), the difference between them served as a measure of delayed hypersensitivity. The observed reaction had the following characteristics. It began at about 3–6 hr, reached a peak at 24 hr, and subsided gradually over the next 4 days. Histologically the cellular infiltrate was predominantly mononuclear in character and reactivity could be transferred to normal recipients with 10^8 spleen cells from a sensitive donor, but not with immune serum in amounts equivalent to the circulating blood volume. The reaction thus fulfilled all of the criteria by which delayed-type hypersensitivity is recognized.

General Procedure.—At intervals after primary and secondary infection, BCG mice from each experimental group were used in a variety of tests: (*a*) The level of delayed hypersensitivity was measured in 10 mice injected with PPD 24 hr previously. (*b*) Five mice were injected intraperitoneally with the suspension of *S. typhimurium* and *M. smegmatis* to measure

bactericidal activity in peritoneal macrophages. (*c*) 10 min later, approximately 10^5 *L. monocytogenes* in 0.2 ml BSS were inoculated intravenously into the five mice from (b) and an additional five mice. (*d*) 5 min after the challenge with *L. monocytogenes*, the 5 mice of (*b*) were bled from the retro-orbital venous plexus and killed by cervical dislocation. The peritoneal cavity was washed out (bactericidal activity of peritoneal cells) and the liver and spleen removed to estimate the viable counts of *L. monocytogenes* and BCG. (*e*) The remaining five mice were killed 24 hr later and the liver and spleen were removed to obtain viable counts of *L. monocytogenes*.

The viable count of *L. monocytogenes* or BCG from each mouse organ or blood was transformed to the $\log_{10}$ and the results from each group of mice were expressed as the geometric mean value to the $\log_{10}$. Unless otherwise stated, all means quoted refer to groups of five mice, except for the delayed hypersensitivity tests which were done on groups of 10 mice.

RESULTS

Host Response to Primary Infection with BCG

Three groups of mice were used: a group of unvaccinated controls (group I), and two groups of vaccinated mice which received low (group II) and high (group III) doses of BCG intravenously. Viable counts showed that the latter groups received 4×10^3 and 4×10^6 viable BCG, respectively. Batches of five mice from each group were tested for resistance to *Listeria* challenge at 3 day intervals during the first 15 days of the experiment, at weekly intervals until the 35th day, and finally on the 56th day.

The mean viable counts of *L. monocytogenes* obtained from the liver, spleen, and blood of mice 5 min after intravenous challenge are shown in Table I. The mean derived from each group of BCG-infected mice was compared with the corresponding mean from the control mice using the *t* test.

As early as the 6th day after infection with BCG, there was a highly significant decrease ($P < 0.01$) in the number of viable *L. monocytogenes* in the blood of the high dose mice of group III when compared with controls. The increased rate of clearance of *Listeria* in these animals reached a maximum on the 15th day, after which there was a gradual waning of the effect. The viable counts of *L. monocytogenes* in the blood of control and low dose mice, though more similar, were usually lower in the latter. The differences were seldom significant (Table I), but comparison of the means of the pooled data derived from the whole experiment did yield a significant difference ($P < 0.05$). This confirmed the impression that mice receiving the low dose of BCG also cleared *L. monocytogenes* from the blood somewhat more efficiently than normal controls.

It is evident from Table I that there was little difference between the control mice of group I and low dose mice of group II with respect to the numbers of *Listeria* implanted in the liver, but the viable counts from the livers of high dose mice (group III) tended to be lower than in the livers of control mice and such differences were sometimes significant. However, the difference between

the means of the pooled data was small and nonsignificant, suggesting that the observed differences were due to experimental variation. Similarly, there was no material difference between the counts of *L. monocytogenes* in the spleens of the control and low dose mice. However, commencing on the 6th day, the counts from the spleens of the high dose mice were invariably higher than those in controls, reaching a maximum difference on the 15th day. Although the mag-

TABLE I

Primary Infection with BCG: Distribution of L. monocytogenes in Blood, Liver, and Spleen of Mice 5 Min after Intravenous Challenge with Listeria

Day of *Listeria* challenge after BCG infection	Mean viable counts of *L. monocytogenes* ($\log_{10}$)								
	Blood			Liver			Spleen		
	Group			Group			Group		
	I	II	III	I	II	III	I	II	III
	Normal controls	4×10^3 BCG	4×10^6 BCG	Normal controls	4×10^3 BCG	4×10^6 BCG	Normal controls	4×10^3 BCG	4×10^6 BCG
3	3.27	3.20	2.96	5.05	5.04	5.12	3.88	3.81	3.89
6	3.63	3.31	2.76*	5.28	5.21	5.18	3.98	4.04	4.22‡
9	3.23	3.87‡	2.47*	5.55	5.54	5.57	4.42	4.34	4.58‡
12	3.73	3.57	2.48*	5.48	5.55	5.45	4.46	4.29	4.67
15	4.01	3.76	2.41*	5.26	5.24	5.22	4.12	4.21	4.60*
21	3.87	3.09*	2.43*	5.58	5.53	5.42*	4.35	4.47‡	4.59*
28	3.66	3.21	2.73*	5.28	5.26	5.15‡	4.39	4.10	4.35
35	3.83	3.35	2.97*	5.56	5.52	5.26	4.44	4.41	4.65
56	—	—	—	5.05	4.94‡	4.96‡	3.62	3.90‡	3.99*
Means of pooled data.....	3.63	3.43‡	2.66*	5.34	5.31	5.26	4.18	4.18	4.39‡

* $P < 0.01$ ⎫ When group I was compared
‡ $P < 0.05$ ⎭ with group II or group III in t tests.

nitude of these differences was variable, the means derived from the pooled data did differ significantly ($P < 0.05$). These small differences in clearance rates and initial distribution of organisms between liver and spleen, though not very meaningful in themselves, were a minor reflection of the much larger differences found in the subsequent growth of *Listeria* in mice belonging to different groups.

The levels of host resistance to *Listeria* in the experimental groups was estimated by comparing the increase in the number of *Listeria* during a 24 hr period in the livers and spleens of control mice with the corresponding increase found in the BCG-infected mice. For each group the mean viable count ($\log_{10}$) of *L. monocytogenes* obtained at 5 min was subtracted from the corresponding 24

TABLE II

Mean Viable Counts of L. monocytogenes in the Livers and Spleens of Normal and BCG-Infected Mice, with Indices of Resistance

Day of *Listeria* challenge after BCG infection	Group*	Liver					Spleen	
		Mean viable counts of *L. monocytogenes* ($\log_{10}$)		Geometric increase in 24 hr	Index of resistance between groups: I–II I–III	*P*	Index of resistance between groups: I–II I–III	*P*
		5 min	24 hr					
3	I	5.05	7.03	1.98	—		—	
	II	5.04	7.12	2.08	−0.10	N.S.‡	−0.37	N.S.
	III	5.12	6.70	1.58	0.40	<0.05	0.19	N.S.
6	I	5.28	7.35	2.07	—		—	
	II	5.21	7.25	2.04	0.03	N.S.	0.04	N.S.
	III	5.18	6.09	0.91	1.16	<0.01	1.01	<0.01
9	I	5.55	7.68	2.13	—		—	
	II	5.54	7.15	1.61	0.52	N.S.	0.10	N.S.
	III	5.57	4.56	−1.01	3.14	<0.01	2.75	<0.01
12	I	5.48	7.62	2.14	—		—	
	II	5.55	6.74	1.19	0.95	0.01	0.14	N.S.
	III	5.45	4.04	−1.41	3.55	<0.01	3.13	<0.01
15	I	5.26	7.25	1.99	—		—	
	II	5.24	6.21	0.97	1.02	<0.01	0.44	<0.05
	III	5.22	4.04	−1.18	3.17	<0.01	3.46	<0.01
21	I	5.58	7.43	1.85	—		—	
	II	5.53	6.06	0.53	1.32	<0.01	0.64	<0.01
	III	5.42	5.34	0.08	1.93	<0.01	2.35	<0.01
28	I	5.28	7.51	2.23	—		—	
	II	5.26	6.54	1.28	0.95	<0.01	0.40	N.S.
	III	5.15	5.99	0.84	1.39	<0.01	1.61	<0.01
35	I	5.56	7.54	1.98	—		—	
	II	5.52	6.54	1.02	0.96	<0.01	0.58	<0.01
	III	5.26	5.94	0.68	1.30	<0.01	1.66	<0.01
56	I	5.05	6.59	1.54	—		—	
	II	4.94	5.88	0.94	0.60	<0.05	0.65	<0.01
	III	4.96	5.22	0.26	1.28	<0.01	1.71	<0.01

* Group I, normal controls; group II, 4×10^3 BCG; group III, 4×10^6 BCG.

‡ N.S. Statistically nonsignificant, $P > 0.05$.

hr value, to give the geometric increase (or, in some cases, decrease) in the number of *Listeria* during the 24 hr period. The geometric increases calculated for mice of the two infected groups were then separately subtracted from the corresponding control values to provide a measure of host resistance called the index of resistance. The method of calculating the indices of resistance from the liver data is demonstrated in Table II. The corresponding spleen results are expressed as indices of resistance only, omitting the mean viable counts. The statistical significance of each index of resistance was tested by estimating its departure from zero by an analysis of variance.

As early as the 3rd day after infection with BCG, the index of resistance for the livers of the high dose mice of group III was 0.40 log units ($P < 0.05$), and increased progressively to a peak of 3.17 log units on the 12th day. The index declined rapidly between the 15th and 28th days to 1.39 log units, at which level it remained fairly steady until the end of the experiment (Fig. 1, middle panel). Resistance to *L. monocytogenes* developed a little more slowly in the spleen. Significant resistance first appeared on the 6th day, attained a maximum value of 3.46 log units on the 15th day, and then fell rapidly in parallel with the liver indices (Fig. 1).

Host resistance in low dose mice of group II developed much more slowly and did not differ significantly from normal until the 12th day in the liver, and the 15 day in the spleen (Fig. 1 and Table II). Peak resistance was reached on the 21st day, but the indices of 1.32 and 0.64 log units in liver and spleen, respectively, were much lower than those found in the high dose mice of group III. Despite this, a small but significant level of resistance persisted until the 56th day.

The growth curve of BCG in the livers and spleens of mice infected with the larger dose are shown in the upper panel of Fig. 1. The highest counts were obtained on the 12th day of infection, corresponding to the peak of resistance to *L. monocytogenes*. Subsequently, the number of viable BCG declined as resistance to *L. monocytogenes* was waning. Because of technical errors the BCG counts in mice infected with the lower dose were fragmentary, and are not reported here.

An in vitro method, other than that described in Materials and Methods, was used to test the bactericidal activity of peritoneal macrophages from the 3rd to the 12th day of the experiment. It was found to be unsatisfactory so that data for this period are not available. However, the assay procedure using a combined inoculum of *S. typhimurium* and *M. smegmatis*, which was adopted on the 15th day, did not reveal any significant differences from normal in the macrophages of mice infected with either dose of BCG, even though the 15th day corresponded to the time of peak resistance in the liver and spleen. In keeping with this finding, at no time did the peritoneal macrophages show any of the morphological changes which are associated with an increase in their microbicidal activity (see later section).

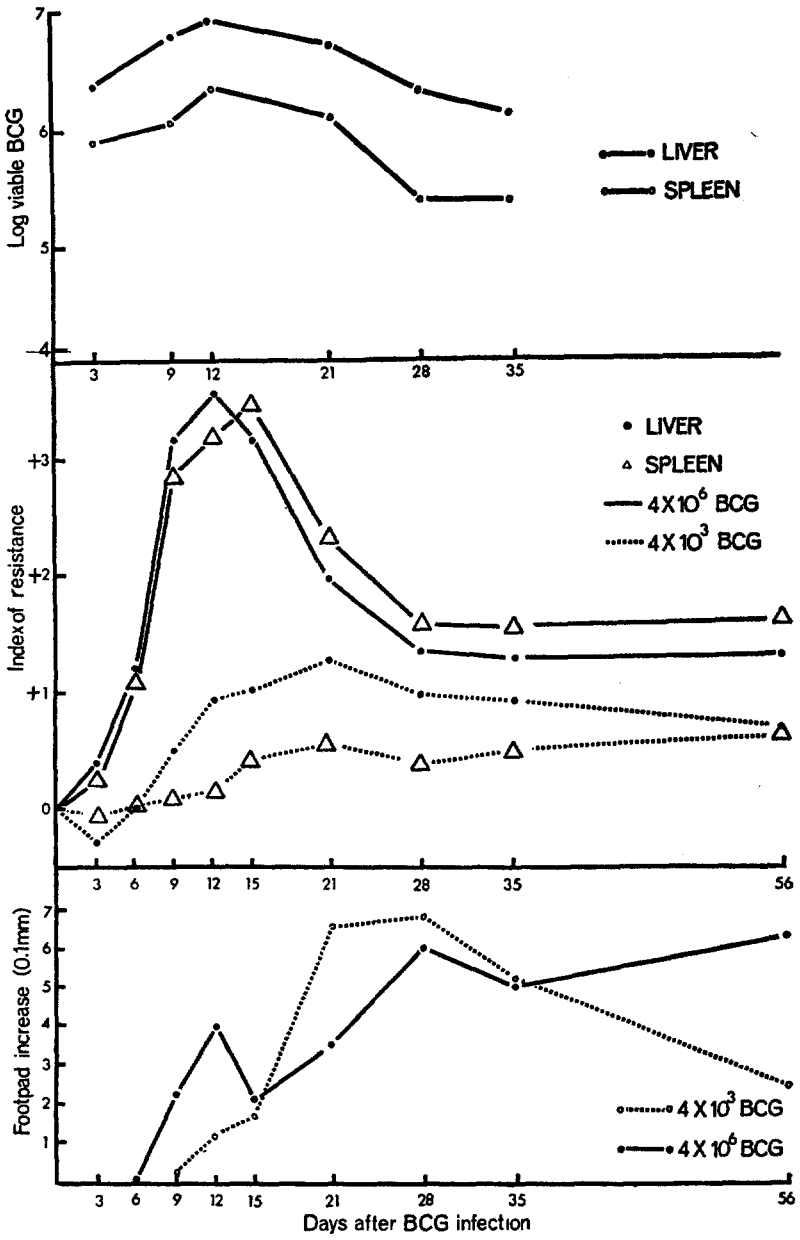

FIG. 1. *Top:* Growth curves of BCG in the livers and spleens of mice given 4×10^6 viable BCG intravenously. *Middle:* Curves depicting the progressive changes in the index of resistance to *L. monocytogenes* in the livers and spleens of mice during a primary infection with BCG at two dose levels. *Bottom:* Levels of delayed hypersensitivity (expressed as increase in foot thickness 24 hr after injection of PPD) found in the BCG infections depicted above. Each point represents the mean of a group of 5 mice (10 in the case of footpad tests).

The development of delayed hypersensitivity is depicted in the bottom panel of Fig. 1. Mice infected with the larger dose of BCG exhibited a biphasic response. Hypersensitivity increased steadily until the 12th day when the number of viable BCG and resistance to *L. monocytogenes* were near their maxima in the liver and spleen. As the BCG population in these organs began to decline, hypersensitivity decreased temporarily before returning to a sustained high level. Mice receiving 4×10^8 BCG became equally hypersensitive without showing a period of desensitization, but a high level of hypersensitivity was not sustained in these mice. It had fallen to a low ebb by the 55th day when the remaining mice of this group were introduced into the reinfection experiment which follows.

Response to Reinfection with BCG (Experiment 1)

On the 55th day the remaining mice which had been infected with the lower dose of BCG were divided into two equal groups, one of which was reinfected intravenously with 5×10^7 viable BCG. At the same time, half of a group of normal mice received the same large dose of BCG. This created 4 groups: group I, normal controls; group II, mice which had received only the first injection of BCG 55 days previously; group III, infected controls which received only the reinfecting dose of BCG; and group IV, which received both the immunizing and reinfecting doses of BCG. Mice from each group were used at daily intervals over the next 5 days to measure the level of resistance to *L. monocytogenes* in liver and spleen, the bactericidal activity of peritoneal macrophages, and the level of delayed hypersensitivity to PPD.

Resistance to *L. monocytogenes* in the livers and spleens of BCG-infected mice (groups II, III, and IV) was again expressed as indices of resistance relative to normal mice (group I). In addition, the resistance of mice which had been twice infected with BCG (group IV) was expressed as indices of resistance relative to mice which had received only the small immunizing dose of BCG (group II), as shown in columns 4 and 6 of Table III.

The indices of resistance in the livers and spleens of the immunized mice of group II were slightly increased throughout the experiment (Table III). On day 2, the indices of resistance in the livers of the infected controls (group III) and the reinfected mice of group IV were significantly increased, and continued to rise in parallel until the end of the experiment (Fig. 2A). When the liver indices between groups I and III and between groups II and IV were compared (Table III), it appeared that prior immunization with BCG had conferred no advantages.

Increased resistance to *L. monocytogenes* in the spleen was detected in the infected control mice of group III on day 1, increased rapidly to 2.47 log units on day 2, and then remained fairly steady at approximately the same level throughout the 3rd, 4th, and 5th days (Fig. 2C). Resistance in the spleens of the reinfected mice of group IV increased at approximately the same rate as in infected controls (group III) for the first 3 days, then continued to rise, so that on the 4th and 5th days the indices of resistance between groups II and IV were clearly greater than between groups I and III (Table III).

The results obtained from testing the bactericidal activity of peritoneal macrophages were evaluated by analysis of variance. The significance of the differences between the normal and infected controls, and between the im-

TABLE III

Indices of Resistance in Livers and Spleens of Mice after Primary and Secondary Infections with BCG (Experiment 1)

Day of *Listeria* challenge after first (or second) BCG infection	Group*	Liver		Spleen	
		Index of resistance calculated between groups		Index of resistance calculated between groups	
		I–II I–III I–IV	II–IV	I–II I–III I–IV	II–IV
56 (1)	II	0.60‡	—	0.65‡	—
	III	0.20	—	0.90§	—
	IV	0.46	−0.14	0.97§	0.32
57 (2)	II	0.83§	—	0.67‡	—
	III	2.16§	—	2.47§	—
	IV	2.41§	1.58§	2.50§	1.83
58 (3)	II	0.59‡	—	0.59‡	—
	III	2.57§	—	2.20§	—
	IV	2.71§	2.12§	2.54§	1.95§
59 (4)	II	0.81§	—	0.64‡	—
	III	2.58§	—	2.17§	—
	IV	3.21§	2.40	3.66§	3.02§
60 (5)	II	0.18	—	0.06	—
	III	3.42§	—	2.77§	—
	IV	3.92§	3.74§	3.83§	3.77§

* Group II, 4×10^3 BCG; group III, 5×10^7 BCG; group IV, 4×10^3 BCG + 5×10^7 BCG.

‡ P 0.05.

§ P 0.01.

munized and reinfected mice was tested at the 5% level as indicated in Table IV. The mean corrected survival of *S. typhimurium* was approximately 50% in normal mice on each of the first 4 days. This level of survival is usually found in normal mice, so that the low value of 33.1% obtained on the 5th day is attributable to experimental variation. There was no significant variation between the four mouse groups on the first day. The survival of *S. typhimurium* in reinfected mice (group IV) on the 2nd day was 18.8%, which differed sig-

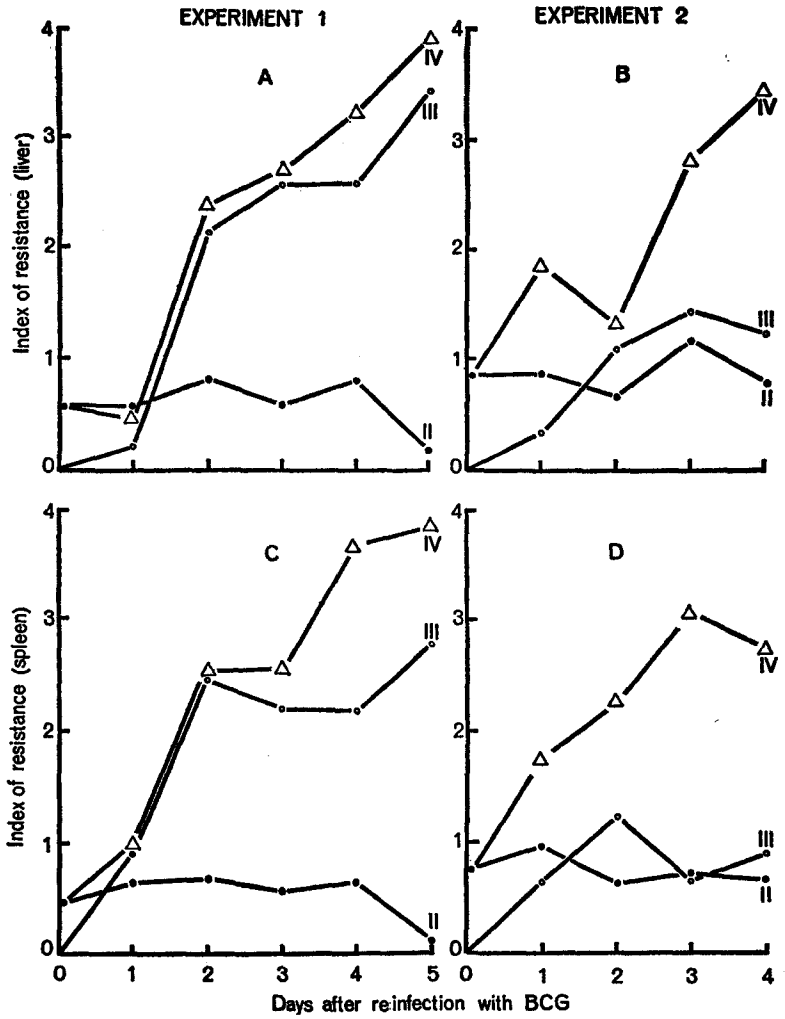

FIG. 2. The results of two experiments showing progressive changes in the index of resistance to *L. monocytogenes* in livers and spleens of mice during primary and secondary infections with BCG. *Experiment 1.* A and C show the resistance of liver and spleen in mice immunized with 4×10^8 BCG 55 days prior to day 0 (group II); infected with 5×10^7 BCG on day 0 (group III); or immunized as in group II and reinfected on day 0 with 5×10^7 BCG (group IV). *Experiment 2.* B and C show resistance of liver and spleen in mice which had been: immunized with 1×10^4 BCG 23 days prior to day 0 (group II); infected with 1×10^7 BCG on day 0 (group III); or immunized with BCG as in group II and reinfected on day 0 with 1×10^7 BCG (group IV).

nificantly from the values obtained in the normal and infected controls but not from that found in the immunized mice of group II. Between days 3 and 5, however, the differences between the immunized and the reinfected mice became statistically significant. By contrast, the peritoneal cells of infected control mice (group III) showed no increase in microbicidal activity until day 5.

At the beginning of the experiment, the animals of group II had been infected with BCG for 55 days. By this time, the level of delayed hypersensitivity in them had fallen to a low level; the tuberculin test produced a mean increase in footpad thickness of 0.24 mm. The injection of 5×10^7 BCG into these mice

TABLE IV

Survival of S. typhimurium in the Peritoneal Macrophages of Mice after Primary or Secondary Infection with BCG (Experiment I)

Day after first (or second) infection with BCG	Mean corrected survival of *S. typhimurium*				Comparison of means between groups	
	Group I	Group II	Group III	Group IV		
	Normal	4×10^8 BCG	5×10^7 BCG	$4 \times 10^8 + 5 \times 10^7$ BCG	I-III	II-IV
	%	%	%	%	%	%
56 (1)	50.7	32.6	34.0	42.1	16.7	−9.5
57 (2)	41.2	31.8	35.2	18.8	6.0	13.0
58 (3)	52.3	48.2	51.4	20.8	0.9	27.4*
59 (4)	50.8	33.4	48.0	20.0	2.8	13.4*
60 (5)	33.1	26.5	11.5	8.4	21.6*	18.1*

* $P < 0.05$.

resulted in transient desensitization to PPD on days 1 to 3, but an enhanced response was obtained in those tested on day 4 (mean increase of 0.39 mm). The same dose of BCG produced no detectable hypersensitivity in previously uninfected mice during the course of the experiment.

In the foregoing experiment, the vaccinated animals enjoyed no striking advantage over normal controls in the rapidity of onset or magnitude of the resistance generated in response to a large inoculum of BCG. In the light of previous experience (2, 6), it was anticipated that prior exposure to BCG would have produced both an acceleration and an enhancement of the resistance evoked by the second injection. Three factors may have combined to produce this unexpected result. (*a*) The immunized mice may have lost most of their capacity to respond anamnestically to reinfection, corresponding to the reduction in their reactivity towards PPD. (*b*) The dose of organisms used in the reinfecting inoculum was so large that it may have produced in normal mice a rapid and maximum response which could not be exceeded even by previously vaccinated mice. (*c*) It is conceivable that a large dose of BCG contributes a strong,

nonimmunological stimulus to host resistance which could overshadow the resistance generated by the specific immunological response to BCG.

In view of the above considerations two additional experiments were performed. In the first, a smaller reinfecting dose of BCG was used, and the animals

TABLE V

Indices of Resistance in Livers and Spleens of Mice after Primary or Secondary Infection with BCG (Experiment 2)

Day of *Listeria* challenge after first (or second) BCG infection	Group*	Liver		Spleen	
		Index of resistance calculated between groups		Index of resistance calculated between groups	
		I-II I-III I-IV	II-IV	I-II I-III I-IV	II-IV
24 (1)	II	0.88‡	—	0.96‡	—
	III	0.33	—	0.64‡	—
	IV	1.83‡	0.95‡	1.74‡	0.80‡
25 (2)	II	0.49‡	—	0.64‡	—
	III	1.08‡	—	1.23	—
	IV	1.30‡	0.81‡	2.24	1.60§
26 (3)	II	1.17‡	—	0.74‡	—
	III	1.42‡	—	0.65	—
	IV	2.79‡	1.62‡	3.06	2.32‡
27 (4)	II	0.76‡	—	0.69‡	—
	III	1.22‡	—	0.91	—
	IV	3.40‡	2.64‡	2.72	2.03‡

* Group I, normal controls; group II, 10^4 BCG; group III, 10^7 BCG; group IV, $10^4 + 10^7$ BCG.

‡ $P < 0.01$.

§ $P < 0.05$.

of the vaccinated group were introduced into the experiment at a time when delayed hypersensitivity was at its height. The second experiment was designed to examine the possibility that a large inoculum of BCG causes nonimmunological stimulation of host resistance.

Host Response to Reinfection with BCG (Experiment 2)

A large group of mice were infected intravenously with 10^4 viable BCG. After an interval of 23 days, half of these infected mice and half of a group of normal mice were inoculated intravenously with 10^7 viable BCG to make four groups which corresponded with those of the previous experiment: group I, normal controls; group II, infected with 10^4 BCG 23 days previously; group III, infected with 10^7 BCG; and group IV, infected with 10^4 BCG and rein-

fected with 10^7 BCG. Mice of group II had a high level of delayed hypersensitivity; tuberculin tests produced a mean increase in footpad thickness of 0.67 mm. Mice from each group were tested for *Listeria* resistance at daily intervals for the first 4 days after injection of the reinfecting dose of BCG.

The indices of resistance to *L. monocytogenes* in liver and spleen are shown in Table V. As in the earlier experiment, groups II, III and IV were compared with group I; and group IV was also compared with group II. There was a slight and statistically insignificant increase of resistance to *L. monocytogenes* in the livers of the infected controls of group III on the 1st day. By day 2, this resistance had increased to 1.08 log units, a highly significant level. The indices of

TABLE VI

Survival of S. typhimurium in the Peritoneal Macrophages of Mice after Primary or Secondary Infection with BCG (Experiment 2)

Day after first (or second) infection with BCG	Mean corrected survival of *S. typhimurium*				Comparison of means between groups	
	Group I	Group II	Group III	Group IV		
	Normal	4×10^8 BCG	5×10^7 BCG	$4 \times 10^8 + 5 \times 10^7$ BCG	I–III	II–IV
	%	%	%	%	%	%
24 (1)	59.1	57.2	48.2	27.8	10.9	29.4*
25 (2)	72.2	55.1	73.7	15.2	−1.5	39.9*
26 (3)	65.8	51.7	58.8	14.9	7.0	36.8*
27 (4)	50.2	46.1	78.4	19.7	−28.2*	26.4*

* $P < 0.05$.

resistance then remained fairly steady on days 3 and 4. The indices of resistance in the livers of the reinfected mice of group IV (relative to group I) rose sharply on day 1, and after a slight drop on day 2 continued to increase to a value of 3.40 log units on day 4 (Fig. 2B).

The spleen results were similar to those of the liver. Infected control mice of group III showed an increase in resistance on day 1 which did not rise appreciably higher on subsequent days. By contrast, resistance in the spleens of the reinfected mice of group IV increased steadily during the first 3 days, reaching a peak of 3.06 log units (Fig. 2D). When comparisons were made between the infected (group III) and uninfected (group I) controls, and between the immunized (group II) and reinfected mice (group IV), the index of resistance in mice of the last mentioned group was much greater in both liver and spleen on days 3 and 4 (Table V).

The bactericidal activity of the peritoneal cells of the reinfected mice of group IV was greatly enhanced on the 1st day after the boosting dose of BCG (Table VI). This level of activity was steadily maintained for the remaining 3

days of the experiment. There was no significant variation between groups I, II, and III, except on day 4 when the survival of *S. typhimurium* in the macrophages of mice belonging to the group of infected controls (group III) was

TABLE VII

Indices of Resistance in Livers and Spleens of Mice after Intravenous Challenge with Dead or Living BCG

Day of *Listeria* challenge after BCG infection	Group*	Liver		Spleen	
		Index of resistance calculated between groups		Index of resistance calculated between groups	
		I–II, I–III, I–IV	*P*	I–II, I–III, I–IV	*P*
1	II	−0.17	N.S.	0.63	<0.05
	III	−0.34	N.S.	0.37	N.S.
	IV	0.25	N.S.	0.39	N.S.
2	II	1.00	<0.01	1.93	<0.01
	III	0.74	<0.05	1.81	<0.01
	IV	0.43	<0.05	0.50	N.S.
3	II	1.81	<0.01	1.24	<0.01
	III	1.36	<0.01	0.81	<0.01
	IV	0.42	N.S.	−0.22	N.S.
4	II	2.36	<0.01	1.63	<0.01
	III	1.67	<0.01	0.37	<0.05
	IV	0.73	<0.05	−0.12	N.S.
5	II	3.17	<0.01	2.23	<0.01
	III	1.07	<0.01	0.69	<0.01
	IV	0.30	N.S.	−0.01	N.S.
8	II	3.95	<0.01	4.40	<0.01
	III	0.26	N.S.	0.34	N.S.
	IV	0.01	N.S.	0.39	N.S.

* Group I, normal controls; group II, 7×10^7 live BCG; group III, 7×10^7 live BCG + isoniazid; group IV, 7×10^7 heat-killed BCG.

significantly greater than normal. This odd result may have been due to random experimental variation.

Comparison of the Response to Living and Dead BCG

Four experimental groups of mice were treated as follows: group I, uninfected controls; group II, mice infected intravenously with 7×10^7 viable BCG; group III, mice infected as in II, but given isoniazid in their drinking water, 0.1 mg/ml, beginning 24 hr prior to infection

and continuing throughout the experiment; group IV, mice injected intravenously with the same suspension of BCG after heat treatment at 56°C for 30 min. A viable count on this suspension later revealed that the killing of BCG was incomplete so that each mouse received approximately 10^3 viable organisms along with 7×10^7 killed BCG. Resistance to intravenous

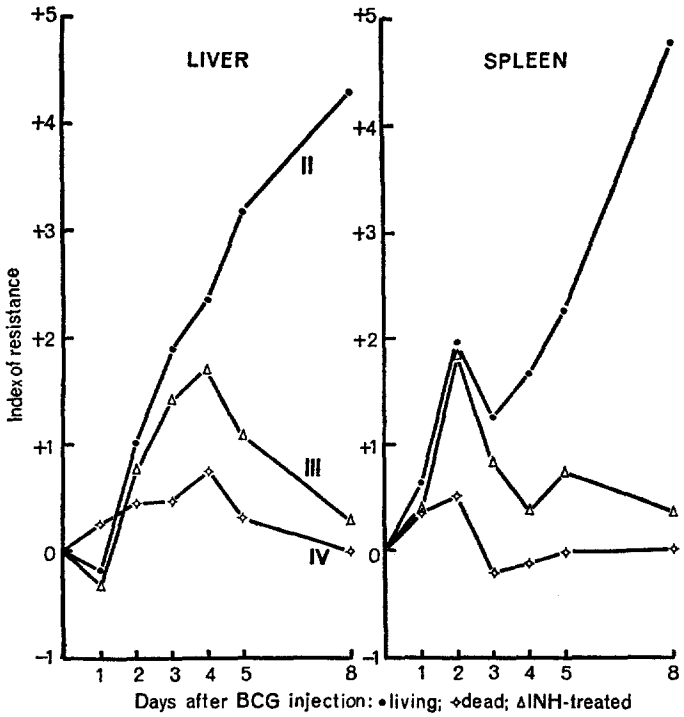

FIG. 3. Progressive changes in the index of resistance to *L. monocytogenes* in the livers and spleens of mice given 7×10^7 BCG on day 0. Those of group II received living BCG; those of group III received the same dose but were treated continuously with isoniazid from one day prior to infection; and those of group IV received the same number of BCG after heat inactivation at 56°C for 30 min.

challenge with *L. monocytogenes* was determined on each group on days 1, 2, 3, 4, 5, and 8 after inoculation with BCG, and delayed hypersensitivity to PPD was measured in 10 mice of each group after 10 days.

The indices of resistance are set out in Table VII and illustrated in Fig. 3. The mice given 7×10^7 viable BCG behaved similarly during the first 3 days regardless of whether they were treated with isoniazid or not. Resistance increased sharply in the spleen for 2 days and then fell on the 3rd day. Thereafter, resistance continued to increase progressively in untreated mice and declined rapidly in those receiving isoniazid. The effects observed in the liver were similar except that resistance increased without interruption in the untreated ani-

mals of group II. In mice inoculated with predominantly heat-killed organisms (group IV), the trends followed those observed in isoniazid-treated animals, but at a much lower level. Resistance in the spleen of group IV animals did not achieve a level of significance and resistance in the liver was only sporadically significant at a low level ($P < 0.05$).

At 10 days after injection of BCG, delayed hypersensitivity was detected in 8 of 10 mice receiving live organisms (mean increase in foot thickness 0.11 mm) and in 4 of the 10 mice treated with isoniazid (0.05 mm); it was entirely absent from mice injected with dead BCG (group IV).

Cytological Changes in the Peritoneal Macrophages of BCG-infected Mice

In animals infected with 4×10^6 viable BCG, the highest levels of resistance to *Listeria* challenge were reached after 12 to 15 days (Fig. 1). At this time, however, the free macrophages of the peritoneal cavity were functionally normal as measured by their capacity to inactivate *S. typhimurium*, and did not differ morphologically from the cells of normal mice. On the other hand, reinfected animals and mice infected for the first time with much larger doses of BCG (10^7–10^8 viable units) showed striking changes in their peritoneal macrophages. The cells from heavily infected mice were seen, by phase-contrast (Fig. 4) and electron microscopy (Fig. 5) to contain greatly increased numbers of mitochondria and lysosomes. These activated macrophages differed from normal cells not merely in structural features; they were also found to spread more rapidly and extensively on glass when cultured in vitro. After 15 min incubation at 37°C, all of the macrophages recovered 2 days after a reinfecting dose of BCG were fully spread (Fig. 6A), whereas the cells of mice which had received only one dose of BCG took 3 hr or more to lose their rounded form (Fig. 6B). Phase-contrast micrographs of macrophages from infected mice also showed increased numbers of translucent vesicles (Fig. 4) which is indicative of increased pinocytic activity (7).

These observations suggest that the morphology of macrophages and their behaviour in vitro faithfully reflect their microbicidal activity in vivo.

DISCUSSION

There is a substantial body of evidence in support of the hypothesis that immunity to tuberculosis is cell-mediated (8) and that this immunity is restricted to the sites of tuberculous inflammation (9). Grogg and Pearse (10) and Dannenberg, Meyer, Esterly and Kambara (11), using histochemical techniques, have shown that macrophages in the vicinity of tuberculous lesions contain more of certain lysosomal enzymes than cells situated more remotely. Furthermore, an increase in lysosomal enzyme content is a consistent feature of cell populations possessing enhanced microbicidal ability (12), although no causal relationship has been established. It follows that the level of host resistance in

tuberculosis depends on a combination of three factors: the number of tuberculous lesions, the size and cellularity of the lesions, and the microbicidal ability of the macrophages within and close to the lesions.

In the present study, a heterologous organism, *L. monocytogenes* was used to measure host resistance in the liver and spleen after primary and secondary infections with BCG. *L. monocytogenes* was chosen because it is a facultative intracellular parasite which multiplies rapidly in vivo and is highly susceptible to inactivation by immune (or microbicidal) macrophages (2, 6). It was hoped that this experimental system would provide a highly sensitive measure of host resistance, and this proved to be the case: indices of resistance as small as 0.60 log units were often highly significant. It can be objected that the resistance to *L. monocytogenes* was not directly analogous to resistance to BCG in these experiments because the immunologically specific components in the microbicidal action of immune macrophages have not been measured. However, leaving aside the requirement of specific opsonins to facilitate the phagocytosis of certain microorganisms (e.g. *Salmonella*), there is no evidence that the microbicidal action of macrophages has an immunologically specific component. On the contrary, there is strong evidence that the microbicidal action of these cells is entirely nonspecific (6, 13, 14).

In this study, it has been shown that after infection with 4×10^6 viable BCG there was a highly significant increase in host resistance to *L. monocytogenes* in both liver and spleen within 6 days, rising to a peak at 12–15 days, and then declining rapidly. There was a corresponding increase in the clearance of *Listeria* from the blood of these mice. The changes after infection with the lower dose of BCG were much smaller, a finding which is consistent with the concept of local immunity. The relationship between resistance and the growth of BCG is of considerable interest. The early phase of resistance to *L. monocytogenes* (3–12 days) coincided with the growth of BCG in liver and spleen, and peak resistance (12–15 days) was reached at a time when multiplication of BCG ceased (Fig. 1). These associations support the hypothesis that resistance to *L. monocytogenes* accurately reflects a parallel resistance to BCG. However, during the period of declining resistance to *Listeria* (15–28 days) there was continuing inhibition of the growth of BCG. An explanation for this apparent anomaly lies in the fact that macrophages are affected systemically only during periods of intense antigenic stimulation (2, 9). As lesions develop, the organisms within them become increasingly confined and their stimulatory effect is largely restricted to phagocytes within the lesions or close by. This concept of local immunity, which was clearly enunciated by Dannenberg (9), helps to explain why resistance to BCG is sustained, while resistance to a *Listeria* challenge declines.

The microbicidal ability of peritoneal macrophage was assayed with opsonized *S. typhimurium* as the target organism. Neither dose of BCG caused any enhancement of bacterial killing by peritoneal macrophages during primary in-

fection. This result is at variance with that obtained in mice infected with *L. monocytogenes*, in whom an increase in microbicidal activity of the peritoneal macrophages was evident from the 4th day of infection and persisted up to a month (15, 16). This discrepancy may merely reflect a difference in the degree of antigenic stimulation since a reinfecting dose of BCG or a large primary intravenous challenge with 10^8 viable BCG regularly produced changes in the peritoneal cells associated with a marked increase in microbicidal ability. This is further evidence that a systemic effect on host macrophages occurs only under conditions of intense antigenic stimulation.

The present observations are reminiscent of less extensive studies made in mice infected with *Brucella abortus* (6). The development of host resistance in *Brucella*-infected mice follows a similar time course, but once established, resistance is more incisively effective against *Br. abortus* than is the antibacterial mechanism developed against BCG. This again implies that BCG is relatively insusceptible to inactivation. As in *Brucella* infection, there was a phase during BCG infection which was associated with partial loss of delayed hypersensitivity. It coincided with the onset of the decline in the number of viable bacteria in the tissues, suggesting that the release of antigen from inactivated bacteria causes desensitization. The transient loss of hypersensitivity after the second intravenous injection of BCG is perhaps analogous.

It was anticipated that host resistance to *L. monocytogenes* would be initiated more rapidly in mice receiving their second dose of BCG than in mice receiving their first dose. In the first experiment, the immunized mice did not respond more rapidly than normal mice to a large BCG challenge (Fig. 2A, 2C), though a high level of resistance was reached by the 4th day. Previous studies had clearly shown that living organisms (*L. monocytogenes*, *Br. abortus*, BCG) will augment the host's defenses at an accelerated rate when injected into homologously immunized mice (2, 6). This discrepancy calls for further comment.

It has long been suspected on circumstantial evidence (17) that the induction of acquired cellular resistance is in some way dependent on the type of immune response which leads to the state of delayed hypersensitivity. Recent studies in this laboratory on the transfer of specific immunity to *L. monocytogenes* from immune donors to normal animals have demonstrated that antibacterial resistance does in fact depend on a state of immunological reactivity which can be transferred with living lymphoid cells but not with serum (18, 19). This indicates unambiguously that acquired cellular resistance arises from a cell-mediated form of immunity, and places it in a category with delayed-type hypersensitivity. It may be relevant, therefore, that the immunized animals of Experiment 1 had lost much of their delayed hypersensitivity to PPD at the time of reinfection. For this reason the experiment was repeated in order to reinfect mice while still at the height of their reactivity to tuberculo-proteins. The level of delayed hypersensitivity was not the only variation introduced into the second reinfection experiment, the other was a reduction in the size of the second

dose of BCG. This appeared necessary in view of the rapidity of onset and the magnitude of the resistance generated during primary infection with a large dose of BCG (Figs. 2A, 2C, group III).

The increase of resistance in the livers and spleens of immunized mice (indices of resistance II–IV, Table V) during the first 2 days was again not conspicuously greater than in mice receiving their first dose of BCG (indices of resistance I–III, Table V). Considered alone, this might suggest that the level of delayed hypersensitivity had no influence on the rapidity with which host macrophages develop increased activity. The experiments in which the microbicidal ability of the peritoneal macrophages was measured provide contrary evidence. In Experiment 1 enhancement of the microbicidal ability of the peritoneal cells was inapparent until the 3rd day, as compared to the first day in Experiment 2. These results are of greater value than those obtained from the liver and spleen, since resistance in the latter organs may have been initiated by the nonimmunological antimicrobial effects of BCG (see next section). On balance, the evidence favors the hypothesis that there is an association between the level of delayed hypersensitivity and the rate at which resistance develops.

A feature of the above experiments was the very rapid onset of resistance in the liver and spleen after a primary BCG infection (Figs. 2 and 3, group III). Although it has been shown that the primary antibody response to some antigens can be detected within 24 hr of challenge (20, 21), it seemed improbable that this would be the case in cell-mediated immunity after BCG infection. The other possibility was that the early phase of host resistance might be due to a nonspecific stimulation of the macrophages in the liver and spleen, quite independent of the immune response to living organisms. During primary BCG infection there was a rapid rise in resistance during the first 2 days which remained steady during days 3 and 4. From day 4, there was a progressive increase of resistance until peak immunity was obtained (Figs. 1, 2, and 3). The biphasic nature of the early response was more clearly seen in the spleen than in the liver. When the nature of the early host response to primary infection was examined experimentally (Table VII), it was found that heat-killed BCG produced no significant increase in host resistance. The response to live BCG was very similar to that obtained in earlier experiments (Figs. 1, 2, and 3). The treatment of infected mice with isoniazid caused an abrupt interruption to the progressive development of resistance on the 3rd and 4th days in the spleen and liver, respectively (Fig. 3). This could be explained by the lag of 72 hr which is sometimes found before the bactericidal action of isoniazid takes effect (22). Alternatively, live tubercle bacilli may contain or elaborate in vivo a heat-labile substance which nonspecifically enhances host resistance.

If living organisms do produce a heat-labile factor which stimulates host resistance nonspecifically, and it continues to be produced by microorganisms growing in vivo, this would imply that the resistance mechanism we have been studying is entirely nonimmunological in origin. This argument can be rejected

because it ignores the findings of earlier studies which showed that cellular resistance to infection is increased (16) or reestablished (2) in previously infected animals only by the microbial species with which the animal was previously vaccinated. These findings, as well as the recent demonstration (18, 19) that the cellular immunity transferrable with spleen cells is specific for the infective agent used to immunize the donor, point strongly to the conclusion that the mechanism of resistance is the outcome of an immunological event. The accelerated onset of resistance in the vaccinated animals of the second reinfection experiment reported in this paper constitutes further evidence of an immunological basis for the resistance to heterologous organisms developed by BCG-infected mice. Moreover, the failure of animals to develop delayed-type hypersensitivity or any significant increase in resistance to a *Listeria* challenge when injected with large numbers of heat-killed BCG points clearly to the involvement of an immunological process in the induction of the cellular changes which make for increased host resistance.

SUMMARY

Heterologous organisms (*L. monocytogenes* and *S. typhimurium*) were used to study the rate of development, magnitude, and persistence of the antimicrobial resistance engendered in mice by vaccination with BCG. These same methods were used to investigate the influence of prior vaccination on the host response to reinfection.

The rate of onset and magnitude of the resistance produced by BCG varied with the vaccinating dose. Increased resistance was detected within 48 hr of injecting large numbers of BCG (approximately 10^8 viable units), but concurrent treatment with isoniazid interrupted its further development. An equal number of heat-killed organisms failed to influence host resistance significantly. The development of tuberculin sensitivity was also dependent upon the continued survival of the immunizing population of BCG.

When vaccinated mice were reinfected with BCG, host resistance in spleen and liver was rapidly augmented to the accompaniment of striking changes in the morphology and microbicidal activity of the peritoneal macrophages. These changes occurred most rapidly in mice with a high level of delayed hypersensitivity at the time of reinfection.

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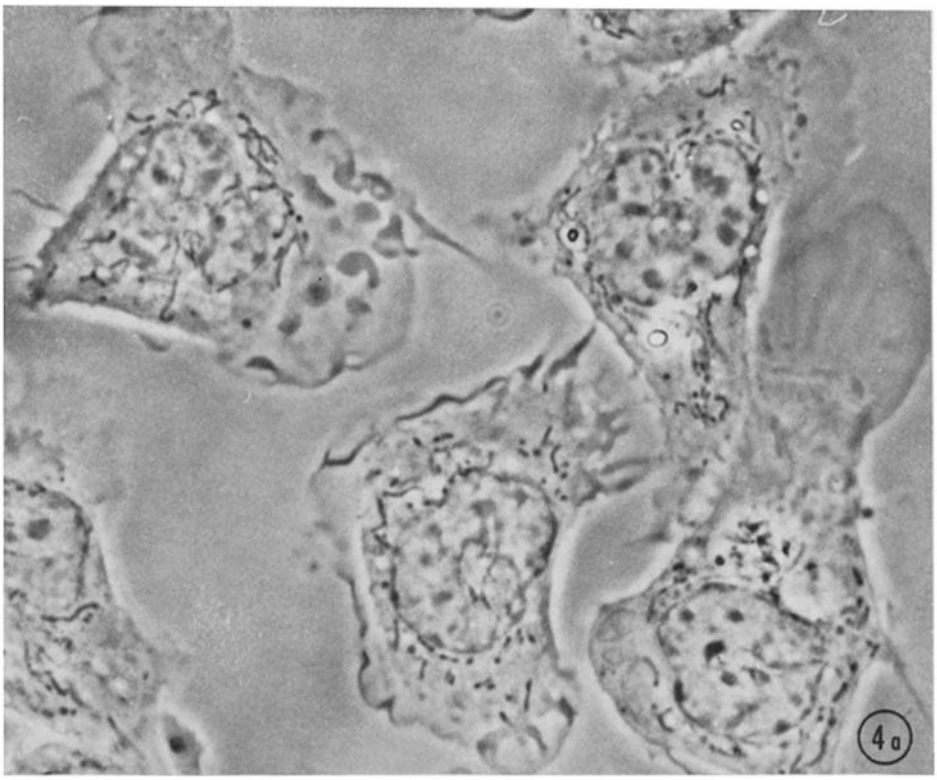
FIG. 4. Phase-contrast appearance of peritoneal macrophages from normal mice (A) and BCG-infected mice (B) after incubation for 24 hr in Hanks' BSS containing 5% fetal calf serum. The cells are conspicuously different in size, tendency to spread, content of mitochondria (filamentious bodies) and lysosomes (phase-dense bodies), and in pinocytic activity (phase-lucent vesicles). Only half of an activated macrophage is contained in the field depicting cells from a BCG-infected animal. These were obtained 14 days after the intravenous injection of 10^8 viable units × 1900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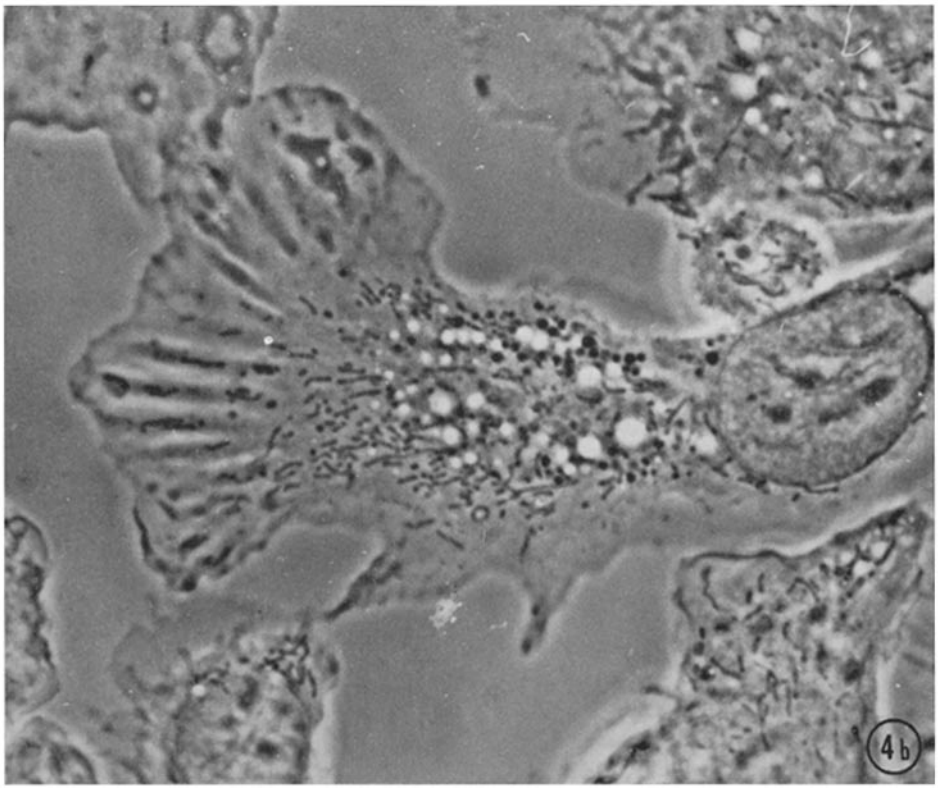
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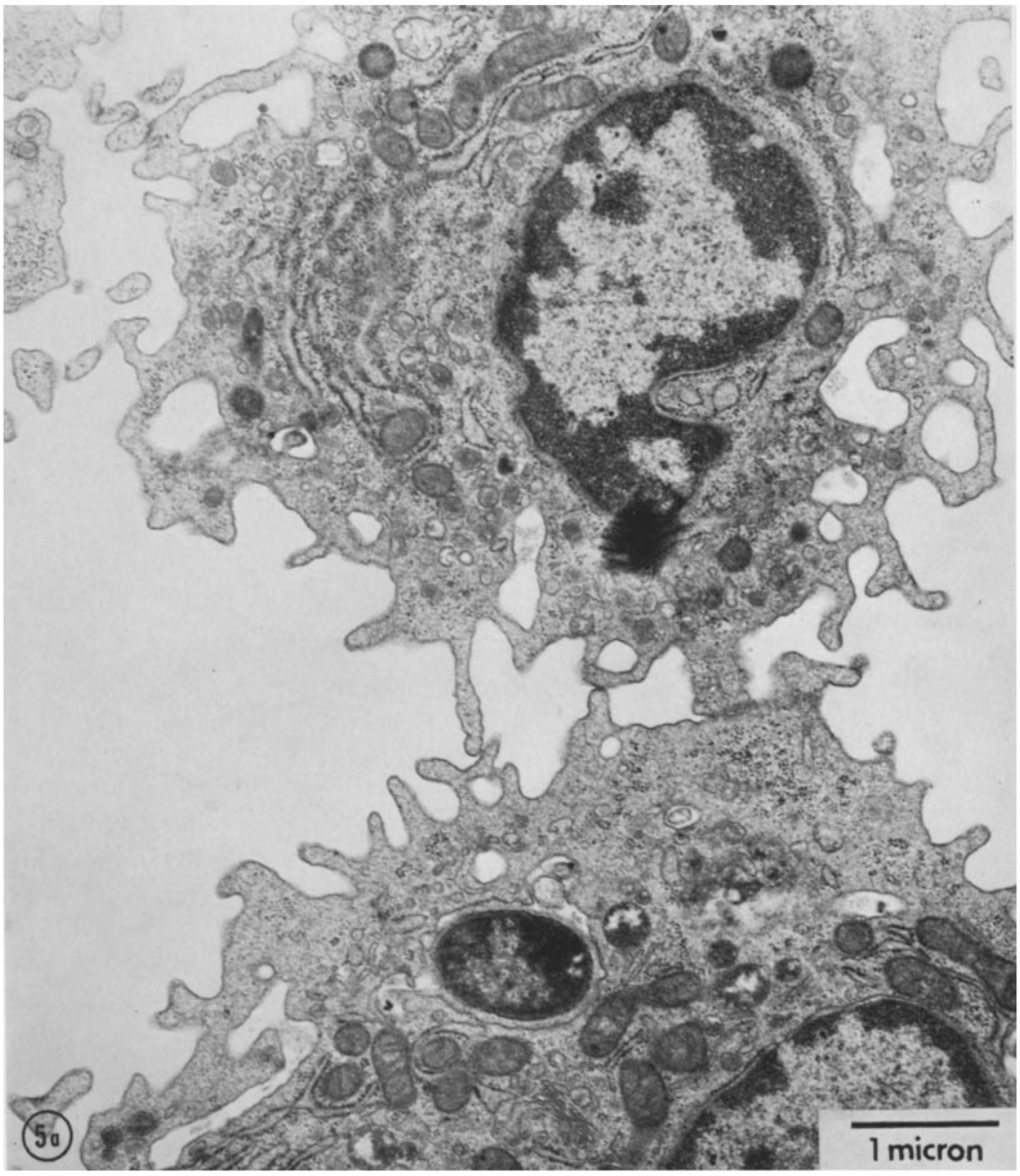
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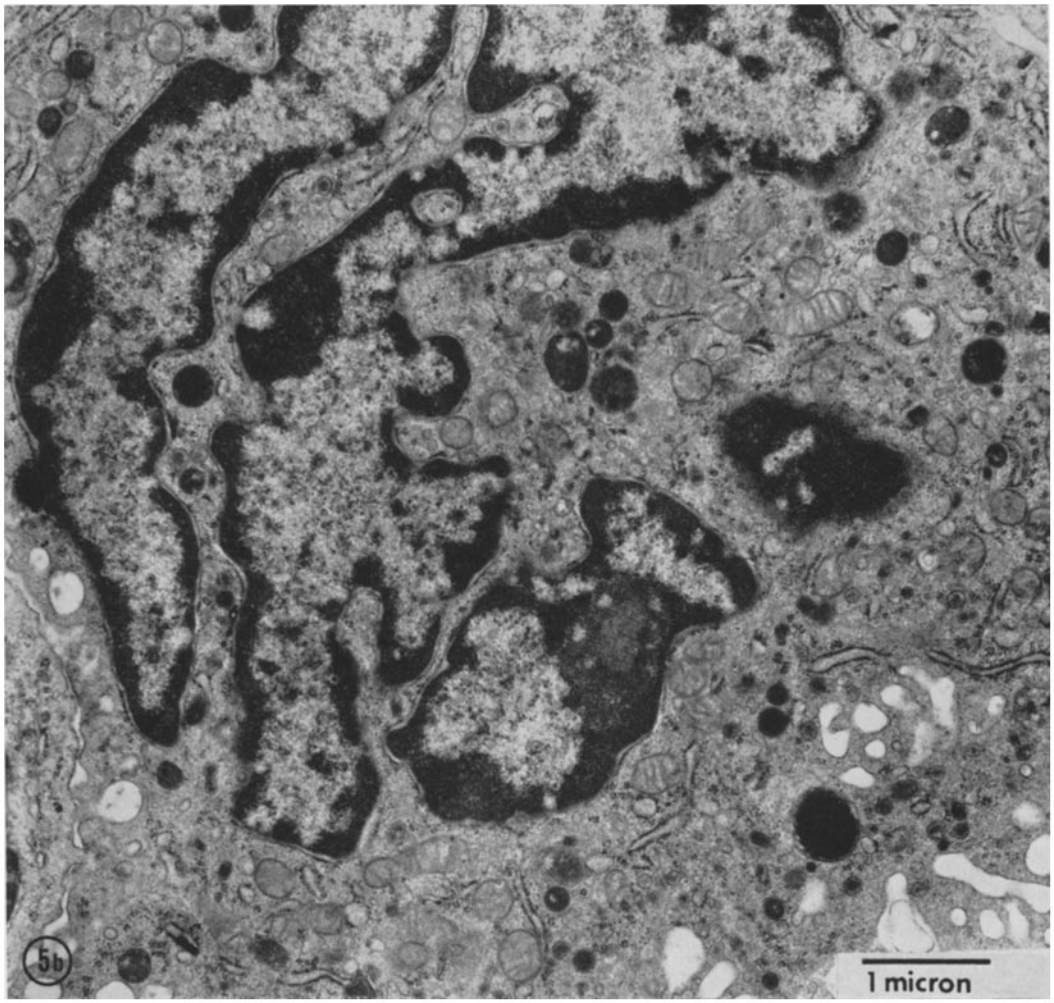
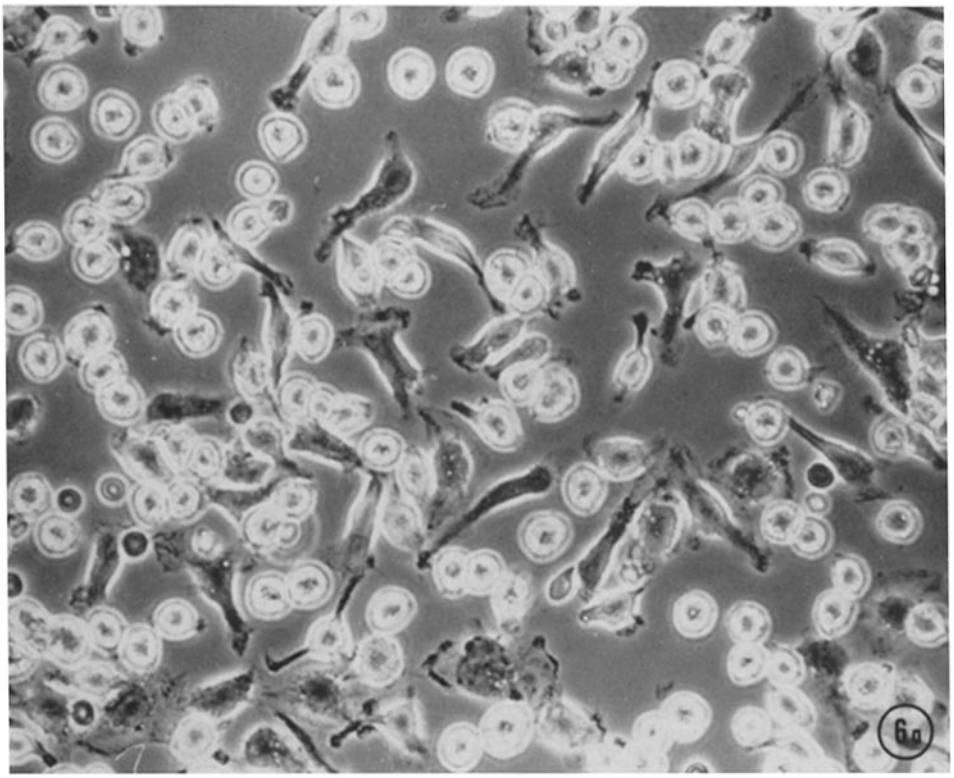


FIG. 5. Ultrastructural features of the peritoneal macrophages illustrated in Fig. 4. The most conspicuous differences are the greater size and content of organelles in the cells from a BCG-infected animal (B) when compared with normal cells (A). One of the latter contains an ingested organism; this was part of a largely negative attempt to demonstrate a greater than normal capacity for digestion on the part of activated macrophages. Glutaraldehyde-osmium tetroxide fixation; uranyl acetate and lead citrate stain. Normal and immune cells are magnified equally.

FIG. 6. Cultural characteristics of peritoneal macrophages from: BCG-infected mice given 1×10^4 BCG 25 days previously (A) and similarly vaccinated animals given a second injection of 1×10^7 BCG 48 hr prior to recovery of cells. Only half of the cells from vaccinated but unchallenged mice had begun to spread into elongated forms when fixed after 3 hr of incubation at 37°C, whereas all the cells of the reinfected animal were spread when fixed after only 15 min of incubation at 37°C. There is also a characteristic difference in the contours of normal and stimulated cells. The latter tend to spread circumferentially. Cultures prepared from animals of Experiment 2 of Fig. 2 and Tables V and VI. (Phase-contrast, × 470).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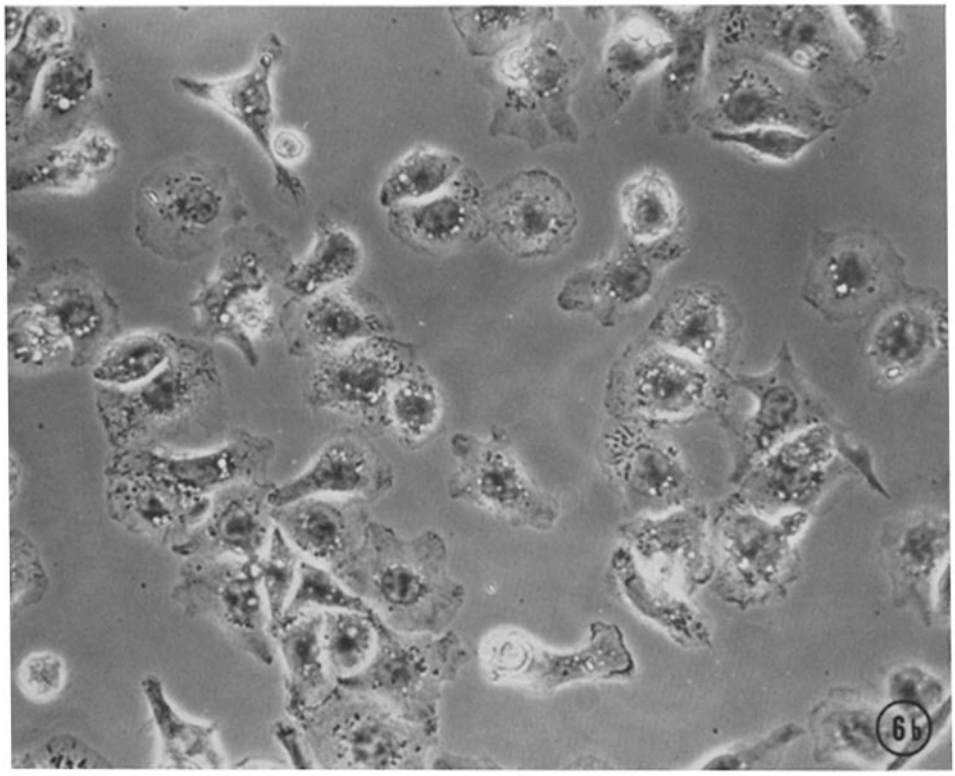
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