INFECTION AND IMMUNITY, Dec. 1975, p. 1295-1306
Copyright © 1975 American Society for Microbiology

Vol. 12, No. 6
Printed in U.S.A.

Host-Parasite Interactions with Peritoneal Macrophages of Mice and Rats In Vitro and In Vivo

WOLF-HELMUT WAGNER

Hoechst AG, Frankfurt (Main), Federal Republic of Germany

Received for publication 7 July 1975

This paper deals with the intracellular multiplication of mycobacteria in peritoneal macrophages from mice and rats immunized with tubercle bacilli or pretreated with Triton WR 1339. If unstimulated macrophages were used, almost unrestricted multiplication of mycobacteria was observed in macrophages from both vaccinated and pretreated hosts after infection of the cells in vitro. Only when the infection of the cells was performed in the peritoneal cavity of vaccinated hosts did the macrophages display a high degree of inhibition. This striking difference in the behavior of macrophages infected in vitro and in vivo is explained by the local inflammation caused by the intraperitoneal infection, which leads to an influx of T-cell mediators. When macrophages from hosts pretreated with Triton WR 1339 were used, inhibition of the multiplication of mycobacteria within cells infected in vitro or in vivo was very slight, though this compound displayed a marked protective effect in the host. Addition of streptomycin to the culture medium caused a strong inhibition of intracellular mycobacteria even in small concentrations; there was no difference between normal and "immune" macrophages. When rats were infected with virulent tubercle bacilli, they were initially fully susceptible to the infection but showed rapid onset of a strong immune response.

For studies of host-parasite interactions, peritoneal macrophages in culture were interacted with *Mycobacteria, Salmonellae, Listeriae, Pasteurellae,* viruses, fungi, protozoa, and tumor cells (for review, see reference 68). Especially the macrophage-tubercle bacillus interactions with respect to resistance to tuberculosis have been studied extensively by many investigators (1, 3, 11, 13, 16, 27, 33, 42, 45-48, 50, 54-56, 62-64, 69, 74).

Most frequently macrophages from the peritoneal cavity of mice have been used in these experiments, although alveolar macrophages differ from peritoneal macrophages by their higher content of lysosomal enzymes and their higher degree of activation (14, 57-59) and therefore appear to be more suitable for such studies.

The aim of the experiments reported here was to investigate the behavior of peritoneal macrophages of mice and rats in vitro and in vivo towards an experimental infection with mycobacteria in relation to acquired cellular immunity or resistance of the host.

For this purpose, mice and rats were immunized and reinfected with mycobacteria; in other experiments animals were treated with Triton WR 1339 in order to enhance resistance to infection. Cultured peritoneal macrophages, both from immunized and pretreated mice and rats, were infected with mycobacteria under different experimental conditions. The intracellular multiplication of bacteria as well as cellular morphology were used to assess the degree of acquired immunity or resistance.

MATERIALS AND METHODS

Animals. Outbred mice of the specific pathogen-free strain HOE NMRKf (SPF 71) or female rats respectively of the specific pathogen-free strain SPRKf (SPF 71) were used.

Mycobacteria. *Mycobacterium tuberculosis* strains H37Rv and H37Ra, *Mycobacterium bovis* strains Ravenel and BCG, and *Mycobacterium smegmatis* were maintained by serial passage in vitro.

Preparation of mycobacterial suspensions. For infection of cell culture, mycobacterial suspensions from fluid Dubos medium were washed repeatedly and the bacterial cultures were dispersed until the supernatant contained mainly single acid-fast bacilli. This was used for infection.

Mycobacterial enumeration in infected animals. Groups of three to four randomly selected mice or rats were killed, and the spleens were removed aseptically and homogenized in 10 ml of sterile saline. Suitable dilutions were plated on PH XII agar; after 4 to 5 weeks, colonies were counted as previously described (75).

Vaccination. Mice and rats were vaccinated intravenously; the macrophages of these animals were considered "immune" macrophages.

Treatment. Triton WR 1339 was used.

Assessment of immunity of the immunized host. Both vaccinated and control mice and rats were infected by intravenous injection of 10^7 viable cells from either strain H37Rv or Ravenel. The effect of this immunization was evaluated by recording survival times and viable counts from the spleens in both groups.

Culture media. Medium TC 199 containing 10% fetal calf serum (FCS) was used for the cultivation of uninfected control cells and for the infection of macrophages in vitro. The culture medium for macrophages of mice consisted of NCTC 109 (40%), beef embryo extract (10%), Hanks balanced salt solution (27%) FCS (10%), liver fraction (10%), glutamine solution 5% (3%), and penicillin (10 μg/ml of medium). The medium for rat cells contained 37% FCS instead of 10%, and only TC 199 was used.

Preparation of macrophage cultures. In general, we used Chang's procedure for the collection of peritoneal cells (7). For uninfected control cells and for the infection of macrophages in vitro, unstimulated peritoneal cavities of 5 to 10 mice were lavaged with 2.5 ml of Hanks solution and subsequently with medium TC 199 containing 10% FCS. In rats, 5 ml was used. Heparin was added at a concentration of 4 U/ml (mice) or 8 U/ml (rats) to the lavage. Generally, we obtained 3 to 6 ml of cell suspension containing approximately 10^6 to 3×10^6 macrophage/ml. Pooled suspensions from several animals were used for preparing the cultures. The pH of the medium was adjusted to 7.2 (mice) and 7.5 to 7.6 (rats). For cultivation of the macrophages, 0.5 ml of cell suspension and 0.5 ml of medium were mixed, and the mixtures were placed in Leighton tubes (16 by 150 mm; Bellco Glass, Inc., Vineland, N.Y.).

The cultures were kept in a normal atmosphere at 37 C. In the case of mouse macrophages, the supernatant fluid was replaced by fresh medium 2 to 3 h after implanting the culture. After 24 h, the cultures were infected and streptomycin was added (experiments 1–4, 13–17, 19). In rat macrophage cultures, the medium was replaced after 3 to 4 days. Where indicated, the cultures were infected at this time and streptomycin was added. Subsequently, the medium was replaced either every 2 days (mice) or once a week (rats). Glassware was carefully washed in acid and water; for cleaning of the cover slips an apparatus for continuous watering was designed which proved advantageous (unpublished data).

Infection of macrophages in vitro. After 24 h (mice) or 3 to 4 days (rats) in culture, the cells were infected, each Leighton tube receiving an inoculum of about 10^6 mycobacteria.

Infection of macrophages in vivo. A 0.2-ml amount of a suspension containing 2×10^7 to 5×10^7 mycobacteria/ml was injected intraperitoneally. In some experiments, macrophages were harvested after 2 h as described above. Measurement of intracellular mycobacterial multiplication was performed as indicated above.

In other experiments, macrophages were harvested 2, 4, 24, and 48 h after infection of the donor animals. After transplanting the cell population to Leighton tubes as indicated above, macrophages were allowed to adhere, and the cover slips were removed and stained 2 h afterwards. In most experiments three to five mice were used for each given time point.

Measurement of intracellular mycobacterial multiplication and macrophage morphology. Two hours after infection and 24, 48 h, and up to several days thereafter, two to three randomly selected cover slips were removed and stained by a combined Ziehl-Nielsen and hematoxylin method. The following were scored: (i) number of infected cells; and (ii) number of intracellular mycobacteria per 100 infected macrophages. For these purposes, the data of two to three cover slips removed at the same time were averaged. More than 20 intracellular mycobacteria was defined as a "cord." Using this procedure, it was possible to follow the trend of the bacterial population. The number of macrophages in infected cultures which represents the cell destruction by multiplying mycobacteria was estimated at the end of each experiment, as was the morphology of individual cells.

RESULTS

Multiplication of M. tuberculosis in uninfected normal macrophages of mice. Within 2 h after implantation, virtually all macrophages had spread. Macrophages could be maintained in cell cultures in good condition up to at least 7 weeks (longest observation period used). During this observation period, there was a noticeable increase in size. Between 84 and 92% of the cells were mononucleated; 6 to 12.5% had two nuclei and 0.8 to 1.0% had three nuclei; less than 1% had four or more nuclei. Mitoses could not be observed.

Multiplication of M. tuberculosis in infected normal macrophages of mice. Apathogenic mycobacteria (*M. smegmatis*) did not multiply intracellularly; the attenuated H37Ra strain multiplied initially after infection but did not lead to cell destruction. Infection with the virulent strain, H37Rv, resulted in rapid multiplication and caused destruction of most infected cells within several days.

Inhibition of intracellular multiplication (H37Rv) in normal macrophages of mice by streptomycin. Concentration of 1.0 μg of streptomycin per ml in the medium did not cause significant inhibition of intracellular multiplication; concentrations of 3.0 μg/ml caused slight, concentrations of 6.0 μg/ml moderate, and concentrations of 10.0 μg/ml and more marked inhibition of mycobacteria within the cells. The observation periods in these experiments were up to 9 days.

Infection of immune macrophages of mice in vitro. The results of these experiments are shown in Table 1. It is obvious that the macrophages from immunized and nonimmunized donors did not differ significantly with regard to

inhibition of intracellular mycobacteria. Neither was there a real difference in the percentage of infected cells in the number of phagocytized bacteria at any given time. However, there was a slight difference in the morphology of infected cells in favor of the immune macrophages, and a longer survival of macrophages from immunized mice in comparison to the controls was seen (Fig. 1). In all these experiments, streptomycin had been added to the cultures in various concentrations (experiments 7 and 20: 20 μg/ml; experiment 4, 5 μg/ml). A typical example is shown in Fig. 1.

Because of these unexpected findings, immunization of the donor was tested in a control experiment (Fig. 2). Immunization with strain H37Rv led to a significant prolongation of survival times of donor animals and a decrease of growth curves of acid-fast bacteria in the spleens of the host under the same conditions where we could not demonstrate a clear-cut difference in the bacteriostatic effect of unstimulated peritoneal macrophages.

In a separate experiment, the influence of streptomycin on infection rate and multiplication of phagocytized mycobacteria within normal and immune macrophages was investigated (Table 2). Addition to the culture medium of this antibiotic at a concentration of 5 μg/ml restricted the intracellular multiplication of mycobacteria with increase of time, but to a similar degree in both normal and immune macrophages.

Infection of immune macrophages of mice in vivo. In striking contrast to the results of infecting immune macrophages in vitro are the results of infection in vivo. Table 3 shows the results of two experiments in which pooled peritoneal washings of normal and immunized mice were infected in vivo. At any given time the percentage of infected cells in the control group was higher than in the macrophages of immunized animals. When the results were evaluated in individual animals, the mean percent-

TABLE 1. *Infection of immune macrophages in vitro (mice)*

Expt no.	Macrophages[a]	Percentage of infected cells (no. of H37Rv cells/100 infected cells)									
		2 h[b]		24 h		48 h		144 h		192 h	
1	I	20	(5.14)[c]	30	(3.57)	51	(4.03)	59	(5.25)		
	C	28	(7.21)	34	(4.20)	44	(5.77)	70	(9.18)		
2	I	16.5	(8.98)	46	(6.11)					48.6	(9.17)
	C	7.0	(2.0)	44	(4.93)					41.5	(7.56)
4	I	5.5	(16.19)	28	(17.62)	52.5	(14.44)	54	(19.03)		
	C	3.5	(15.24)	19.5	(9.31)	38	(7.43)	44.5	(17.62)		

[a] I, Immune; C, control.
[b] Time after infection.
[c] Numbers in parentheses are means.

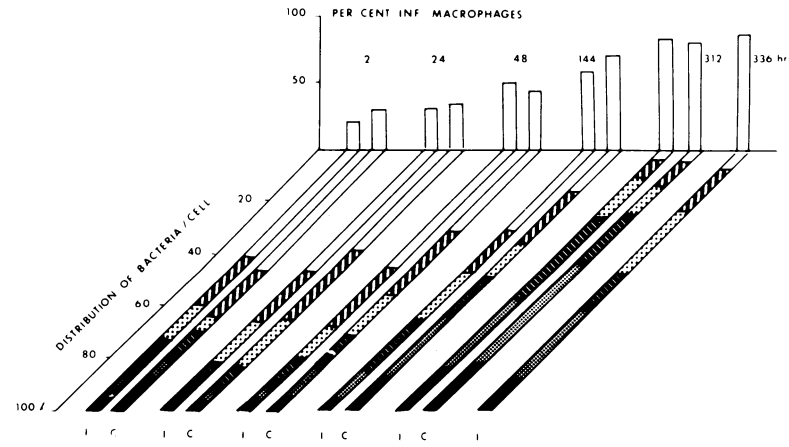

FIG. 1. *Intracellular multiplication of strain H37Rv in macrophages of normal mice. Symbols: □, 1 bacterium/cell; ▨, 2 bacteria/cell; ▩, 3 to 4 bacteria/cell; ▧, 5 to 8 bacteria/cell; ▦, 9 to 16 bacteria/cell; ■, cords. I, Immune; C, controls.*

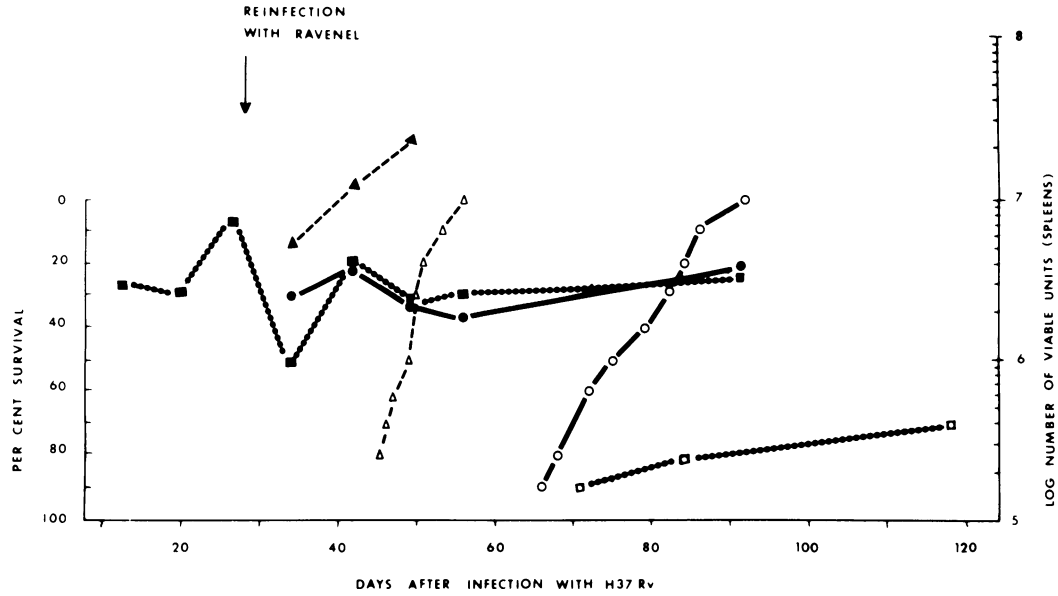

FIG. 2. *Survival rates of mice infected with 10⁶ H 37Rv and reinfected with 10⁶ M. bovis Ravenel, and growth curves of acid-fast bacteria in the spleens of these mice. Controls (only infection with Ravenel), triangles; infection with H37Rv and reinfections with Ravenel, circles; infection with H37Rv, no reinfection, squares. Open symbols are survival rates; closed symbols are growth rates.*

TABLE 2. *Influence of streptomycin on infection rate and mean number of intracellular H37Rv cells within normal and immune macrophages (mice)*

Expt no.	Macrophages	Streptomycin (μg/ml)	Percentage of infected cells (no. of H37Rv cells/100 infected cells)			
			2 h[a]	24 h	48 h	144 h
3	Immune	5	5 (17.87)[b]	22 (13.61)	32.5 (17.09)	42 (15.99)
4	Immune	0	5.5 (16.19)	28 (17.62)	52.5 (14.44)	54 (19.03)
3	Normal	5	1.5	22 (6.59)	29 (12.83)	32 (11.35)
4	Normal	0	3.5 (15.24)	19.5 (9.31)	38 (7.43)	44.5 (17.62)

[a] Time after infection.
[b] Numbers in parentheses are means.

TABLE 3. *Infection of immune macrophages in vivo (mice)*

Expt no.	Macrophages[a]	Percentage of infected pooled cells in peritoneal exudates					
		18 h[b]	20 h	24 h	48 h	72 h	144 h
9	I		28		<1		<1
	C		24		7		8
10	I	5	0		7	5	
	C	10	1.5		10	18	

[a] I, Immune; C, control.
[b] Time after withdrawal of peritoneal fluid.

ages of infected cells in the controls were still higher than in immunized cells (Table 4). It is characteristic of this type of experiment that the number of infected cells decreases with increase of time, presumably because bacteria are carried away by the bloodstream.

Cellular composition of the cell population in the mouse peritoneal cavity. Analysis of cells in the uninfected peritoneal cavity of HOE NMRKf mice showed macrophages to be the

TABLE 4. *Infection of immune macrophages in vivo (mice)*

Expt no.	No. of animals	Macrophages[a]	Percentage of infected cells[b]			
			2 h[c]	24 h	48 h	72 h
11	3	I	2.33 ± 3.2	2.0 ± 1.0[d]	4.3 ± 1.15[d]	2.66 ± 1.16
	3	C	6.5 ± 5.8	14.3 ± 15.9	29.0 ± 8.7	14.5 ± 5.5
12	5	I	2.9 ± 2.37	0.3 ± 0.42[d]	1.14 ± 0.89[d]	
	5	C	7.3 ± 8.3	11.6 ± 9.1	10.6 ± 5.3	

[a] I, Immune; C, control.
[b] Mean ± standard deviation.
[c] Time after infection.
[d] Difference between I and C statistically significant ($P < 0.05$).

predominant cell (~60%); the rest of the cell population consisted mainly of lymphocytes. If mice were infected intraperitoneally, depending on the size of the inoculum and the time after infection, large variations in the counts of macrophages and lymphocytes were found, the ratio of these two cell types being in most cases inverted. Additionally, granulocytes were found in varying numbers.

Growth curves of H37Rv in spleens of normal rats. Rats were infected with strain Ravenel. The bacterial growth curves from spleens (Fig. 3) showed an initial increase followed by a significant decrease in the numbers of viable units, indicating an initial susceptibility of rats to the infection with virulent tubercle bacilli. Obviously, there is a rapid emergence of acquired immunity that prevents rats from succumbing to the tuberculous infection. This is in sharp contrast to the immunological behavior of mice in which infection with a virulent strain in a high infecting dose leads to unchecked multiplication and ultimately to death.

Uninfected normal rat macrophages. For the cultivation of peritoneal macrophages from rats, results were distinctly improved when the pH of the medium was adjusted to 7.5 to 7.6. Under these conditions, the macrophages readily adhered to the cover slip but retained their rounded form for a longer period. Spreading occurred only after 2 to 3 days, during which time the medium was not replaced. Rat peritoneal macrophages have a larger diameter than those of mice; they survive in culture in good condition for several weeks.

Infected normal rat macrophages. Infection with strain H37Rv led to intracellular multiplication and eventually caused destruction of many infected cells. However, in most experiments, intracellular multiplication was slower and the percentage of infected cells lower when compared with corresponding observations in mice.

Infection of immune macrophages or rats in vitro. As in mice (above and Table 1), no significant differences could be found between percentages of infected macrophages and numbers of H37Rv bacteria per 100 infected cells of normal and immunized animals (Table 5).

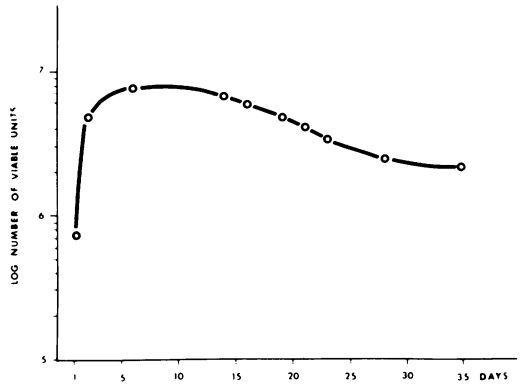

FIG. 3. *Growth curve of H37Rv in the spleens of rats. Inoculum: 10^6 intravenously.*

Infection of immune macrophages of rats in vivo. The results of Table 6 clearly demonstrate significant differences in the percentages of infected macrophages of normal and immunized rats being in good agreement with the corresponding experiments in mice (above and Table 4).

Survival rates and growth curves of H37Rv in spleens of mice pretreated with Triton WR 1339. Figure 4 shows the results of a typical experiment; the survivial rates demonstrate good prophylactic effects of Triton WR 1339.

Infection of Triton-treated macrophages in vitro (mice). In none of the six separate experiments carried out was there a significant difference between controls and macrophages from Triton-treated donors (Table 7).

Infection of macrophages from Triton-treated donors in vivo (mice). With this type of infection, an influence of the above treatment with Triton WR 1339 on the intracellular

TABLE 5. *Infection of immune macrophages in vitro (rats)*

Expt no.	Macrophages[a]	Percentage of infected macrophages (no. of H37Rv cells/100 infected cells)			
		2 h[b]	24 h	48 h	144 h
5	I	1 (13.3)[c]	11 (14.2)	10 (15.1)	6 (8.8)
	C	2 (12.1)	11 (12.1)	10 (17.2)	17 (14.1)
6	I	22 (7.4)	44 (8.4)	85 (13.2)	
	C	28 (7.6)	33 (9.1)	71 (12.5)	
7	I	2	4	17 (3.0)	
	C	1	3	18 (4.3)	

[a–c] See Table 1.

TABLE 6. *Infection of immune macrophages in vivo (rats; experiment 8)*

No. of animals	Macrophages[a]	Percentage of infected cells[b]			
		2 h[c]	24 h	48 h	72 h
5	I	0.02 ± 0.04[d]	1.3 ± 2.6	0.1 ± 0.22[d]	0.3 ± 0.28[d]
5	C	11.8 ± 7.8	4.2 ± 4.0	2.4 ± 2.48	1.2 ± 1.10

[a–d] See Table 4.

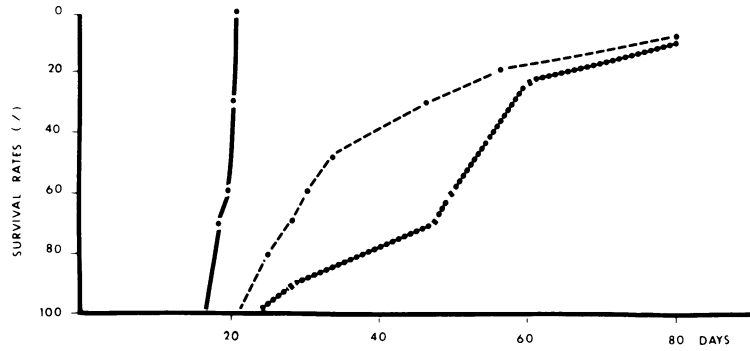

FIG. 4. *Survival rates from mice pretreated with Triton. Symbols: ------, Triton (5 × 500 mg/kg subcutaneously); ●●●●●●, Triton (10 × 500 mg/kg subcutaneously); ——, controls. Infection: M. bovis (Ravenel); infection dose: 10^6 intravenously.*

multiplication of strain H37Rv could not be observed (Table 8).

Infection of Triton-treated macrophages in vitro and in vivo (rats). In both types of experiments, no inhibitory effect of pretreatment with Triton WR 1339 could be demonstrated (Tables 9 and 10).

DISCUSSION

It was the main purpose of these investigations to compare the intracellular multiplication of mycobacteria in peritoneal macrophages from hosts immunized with mycobacteria and from hosts pretreated with Triton WR 1339. These experiments should reveal whether macrophages in tissue culture are a simple tool for testing immunizing procedures that provoke acquired cellular immunity or substances that enhance cellular resistance in the host. Furthermore, these experiments should disclose which experimental conditions are most suitable to demonstrate such effects.

In a previous publication (74), it was found that neither immunization with mycobacteria nor pretreatment with Triton WR 1339 led to a significant activation of unstimulated peritoneal macrophages of mice and rats. In these experiments, slightly better effects in rats than mice were observed. In another series of experiments, these earlier observations were reinvestigated; the experimental conditions were modified. The multiplication of strain H37Rv was tested in normal and immune macrophages from mice and rats. Under different test conditions, completely different and even contradic-

tory results were obtained. If unstimulated peritoneal macrophages from both species were infected in vitro, tubercle bacilli multiplied in immune macrophages almost as rapidly as in those from control animals. However, in some experiments, the macrophages from immunized animals displayed less destruction at the end of the experiment than those from nonimmunized mice and rats (74). Control experiments with mice demonstrated that immunization brought about a significant prolongation of survival times of donor animals and less multiplication of the challenge infection in spleens of mice when compared with nonimmunized animals. In these tests experimental conditions were used, that were similar to those used in

TABLE 7. *Infection of Triton-treated macrophages in vitro (mice)*

Expt no.	Macrophages[a]	Percentage of infected cells (no. of H37Rv cells/100 infected cells)				
		2 h[b]	24 h	48 h	72 h	144 h
13	T		44 (4.6)[c]	60 (6.1)	70 (8.4)	
	C		20 (3.0)	56 (4.8)	—[d]	
14	T			10 (3.8)	12 (5.3)	
	C			12 (4.9)	10 (4.8)	
15	T	30 (4.3)	55 (4.5)	55 (5.4)	70 (7.5)	85 (10.0)
	C	24 (3.8)	50 (3.5)	60 (5.8)	66 (7.6)	50 (12.3)
16	T	50 (4.1)	65 (4.4)	54 (5.8)	69 (7.4)	
	C	40 (3.9)	61 (5.5)	75 (6.1)	73 (7.6)	
17	T	3.8 (11.3)	9 (15.9)	21 (13.0)	19.5 (12.3)	
	C	4 (12.5)	8 (8.5)	25 (6.3)	29 (5.1)	
18	T	25 (3.36)	39 (3.94)	45 (4.02)		60 (8.2)
	C	15 (3.77)	43 (3.28)	46 (3.81)		67 (14.3)

[a] T, Triton treated; C, control.
[b] Time after infection.
[c] Numbers in parentheses are means.
[d] —, Macrophages destroyed.

TABLE 8. *Infection of Triton-treated macrophages in vivo (mice)*

Expt no.	No. of animals	Macrophages[a]	Percentage of infected cells[b]			
			2 h[c]	24 h	48 h	72 h
20	10	T		7.48 ± 8.5	14.8 ± 9.0	10.36 ± 10.0
	10	C		15.46 ± 4.3	13.6 ± 8.5	9.8 ± 9.1
21	5	T	8.0 ± 6.3	12.8 ± 10.1	13.3 ± 8.6	11.3 ± 7.8
	5	C	8.2 ± 6.1	11.0 ± 11.4	10.6 ± 7.5	6.3 ± 7.1
22	5	T			9.6 ± 4.0	
	5	C			6.2 ± 5.3	
23	5	T			14.4 ± 9.3	
	5	C			17.1 ± 11.0	
24	5	T		15.6 ± 7.4	2.4 ± 3.5	5.4 ± 6.3
	5	C		11.6 ± 10.9	9.6 ± 6.8	11.0 ± 6.9
25	5	T	10.2 ± 4.2	23.0 ± 4.6	19.6 ± 10.5	23.8 ± 5.3
	5	C	6.4 ± 5.8	15.0 ± 7.6	20.4 ± 9.7	9.6 ± 8.2

[a] T, Triton treated; C, control.
[b] Mean ± standard deviation.
[c] Time after infection.

TABLE 9. *Infection of Triton-treated macrophages in vitro (rats; experiment 19)*

Macrophages[a]	Percentage of infected cells (no. of H37Rv cells/100 infected macrophages)			
	2 h[b]	24 h	48 h	144 h
T	9 (13.0)[c]		8 (13.5)	23 (16.9)
C	2 (12.1)	11 (12.1)	10 (17.2)	17 (14.1)

[a–c] See Table 1.

TABLE 10. *Infection of Triton-treated macrophages in vivo (rats)*

Expt no.	No. of animals	Macrophages[a]	Percentage of infected cells[b]			
			3 h[c]	24 h	48 h	72 h
26	5	T	19.7 ± 17.0	7.3 ± 5.4	7.8 ± 4.0	7.6 ± 6.2
	5	C	18.8 ± 13.2	1.4 ± 1.9	3.3 ± 3.1	1.8 ± 2.3
27	5	T	21.2 ± 23.4	0.2 ± 0.6[d]	3.3 ± 0.7	3.7 ± 3.7
	5	C	18.2 ± 17.6	4.7 ± 3.6	2.9 ± 2.6	4.7 ± 2.1

[a–c] See Table 8.
[d] Difference between T and C significantly different ($P < 0.05$).

the experiments with macrophages infected in tissue cultures.

If growth curves from the spleens of infected, nonimmunized rats were made, it could be shown that rats initially were fully susceptible to an infection with tubercle bacilli. However, rapid onset of an immune response could be observed which entailed a significant decrease of the number of viable units in the spleen after a short time. These observations agree with the findings of Lefford et al. (40) and Wagner (74) and Kief (34). It has been previously shown that rats, after intravenous injection of human, bovine, and avian tubercle bacilli, develop tuberculous lesions in all the viscera except the kidney, although the infected animals did not show clinical symptoms of the disease. He could demonstrate that at first there is multiplication of tubercle bacilli in the organs, but after different times, dependent on strain and organism, destruction of the bacilli starts in the different organs. In these animals a slight, atypical tuberculin sensitivity developed.

Macrophages of mice and rats differ in that in cells from nonimmunized rats, multiplication of strain H37Rv is slower than in macrophages from mice.

The findings regarding the almost unrestricted multiplication of mycobacteria in unstimulated, immune macrophages of mice agree with the observations of Patterson and Youmans (63). These authors found that the rate of multiplication was the same within normal and immune macrophages. The only effect that could be observed in these experiments in favor of the immune macrophages was a better morphology at the end of the experiment.

In some of our experiments streptomycin was added to the culture medium in varying concentrations. In agreement with previous authors (8, 63), we found that this antibiotic exerts significant inhibition of intracellular mycobacteria even in small concentrations, provided that the observation period is long enough. It could be demonstrated that streptomycin permeates into the cells until an intracellular concentration three to four times that in the medium is reached (4). Streptomycin is concentrated within lysosomes (6, 17, 18, 72); its slow action on intracellular mycobacteria can induce erroneous interpretations. In a critical approach to the technique, Hart (29) dealt with the problems that arise when streptomycin is added to macrophages in culture. If slow-growing organisms are used, extracellular growth can be controlled without this antibiotic, which agrees with our own observations.

Patterson and Youmans (63) came to the conclusion that "the intracellular mycobacteriostatic effect of streptomycin was more pronounced when the peritoneal cells used were obtained from immunized mice than when they were obtained from nonimmunized mice." In our own experiments such an effect was not demonstrable. It can be ruled out that the slight inhibitory effect of immune macrophages infected in vitro as demonstrated by a better morphology of these cells at the end of the observation period is due to an effect of this antibiotic.

When mice and rats were infected intraperitoneally, the macrophages after transferral to the test tubes behaved entirely differently from cells infected in vitro. Those obtained from immunized animals displayed a high degree of inhibition compared with the controls. This effect could be measured if both techniques were used: evaluation of pooled exudates and that of individual exudates. Analogous results were obtained with mice and rats.

The explanation of this striking difference must take into account the fact that the experiments with infection in vitro were performed with unstimulated peritoneal macrophages. Investigations on the local nature of immunity in tuberculosis by Dannenberg (11) and Dannenberg et al. (12, 15) could demonstrate that macrophages enter tuberculous lesions from the bloodstream. Initially, they are in a relatively unactivated state; gradually they become activated and acquire the power to destroy tubercle bacilli. According to these and other authors,

the cellular immunity in tuberculosis is mainly local in nature. If there exists a more extensive infection, a systemic immunity results but the macrophages far from the site of infection are activated only to a low degree. Unstimulated peritoneal macrophages may be regarded as end cells (20). Thus, an intravenous immunization has very little chance of activation of these cells.

This situation is fundamentally altered when a local inflammation is produced. The number of mononuclear phagocytes is being increased at the site of inflammation due to the influx of monocytes (22, 70). When tubercle bacilli are injected into the peritoneal cavity, a severe local inflammation is produced that results in a change of the cell picture; the number of lymphocytes increases, and granulocytes are observed.

Macrophage activation in vivo has been shown to be a result of lymphocyte-mediated immunological processes (36, 43, 44, 49–52). The mediators are specifically sensitized, replicating, short-lived, thymus-derived lymphocytes, i.e., T-cell mediators. This class of lymphocytes confers adoptive immunity to tuberculosis in rats (39). Recently, it could be demonstrated that thymus-dependent lymphoid cells transfer immunity in syngeneic mice. The adoptive immunity in recipient mice was measured in terms of the inhibition of growth of mycobacteria in the liver and spleen after intravenous injection (38). In this context, the findings of Patterson and Youmans (62) must be mentioned, which showed that splenic lymphocytes from mice immunized with viable attenuated mycobacterial cells (H37Ra) are capable of bringing about inhibition of multiplication of virulent tubercle bacilli (H37Rv) within normal peritoneal macrophages of mice in tissue culture. Koster et al. (35–37) and McGregor et al. (44) have shown that thymus-derived lymphocytes preferentially emigrate into peritoneal exudates that have been induced experimentally. Catanzaro et al. (5, 6) described the properties of the resident lymphocytes of the "normal" uninflamed peritoneal cavity. These normal peritoneal lymphocytes were B-cell rich and T-cell poor. Only less than 5% of the peritoneal exudate lymphocytes contained surface immunoglobulin, whereas approximately 50% of the normal peritoneal lymphocytes had surface immunoglobulin. Thus, there is conversion to a B-cell-poor and T-cell-rich population after an experimental inflammation of the peritoneal cavity.

In experiments with mice infected with *Listeria monocytogenes*, North and Spitalny (61) found an accumulation of immunity-mediating T-cells in casein-induced peritoneal exudates. In view of these findings, it appears that macrophages in the unstimulated peritoneal cavity are not, or not sufficiently, activated in order to inhibit intracellular multiplication even if the host is vaccinated with a potent vaccine. Only when a local inflammation is set, permitting an influx of T-cells, does activation of macrophages ensue.

In the experiments described in this paper, the addition of lymphocytes from immune animals to cultures of their macrophages was not done as in studies describing a quantitative in vitro model of cellular immunity in the guinea pig (66, 67), but the findings of Patterson and Youmans (62) and those of Lefford (38) make it highly probable that T-lymphocytes are responsible for the activation of the macrophages.

The role of peritoneal macrophages after a prophylactic dose of Triton WR 1339 remains unclear. This nonionic detergent is a lysosomotropic agent thoroughly studied because of its applicaton in the isolation of hepatic lysosomes (71). Cornforth et al. (9, 10) described the antituberculous effects of Triton WR 1339 and of related nonionic, surface-active preparations. All these substances, even when displaying a high degree of activity in vivo, were practically without tuberculostatic activity in vitro. Thus, it was assumed that Triton WR 1339 exerted its action indirectly, through host mediation. It could be shown that these surfactants can enter macrophages in the living host (41). The inhibition of multiplication of tubercle bacilli within cells which were harvested from animals that had been treated previously with Triton WR 1339 was demonstrated in an early publication (46), but growth of tubercle bacilli in macrophages was unaffected if Triton WR 1339 was added to the tissue culture. It was suspected that this failure was due to the low concentration of the compound tolerated in the culture, and other surfactants were investigated under similar conditions. Macrocyclon, a compound that also belongs to the group of nonionic surface-active polyoxyethylene ethers, when added to the culture medium immediately after infection markedly inhibited the multiplication of strain H37Rv within macrophages (28).

In our experiments described above and elsewhere (27), Triton WR 1339, when applied to the host (mice), displayed a marked protective effect as demonstrated by prolongation of survival times and decrease of the number of viable bacteria in spleen homogenates. In contrast to these findings, neither after infecting macrophages in vitro nor in vivo was there a signifi-

cant difference in the intracellular multiplication of cells harvested from untreated controls or from pretreated hosts. This is a puzzling observation which is not easily explained. In spleen homogenates the number of bacilli decreases after a sufficient dose of Triton WR 1339, and it must be assumed that mycobacteria in these homogenates stem to a large part from crushed macrophages. The pharmakokinetics of Triton WR 1339 were not investigated in the experiments described above. Thus, it can not be ruled out that Triton is only ingested by macrophages of the liver and the spleen and not by the monocytes in the blood stream. Thus the question remains unanswered why peritoneal macrophages, after infection of the peritoneal cavity, are not or not sufficiently activated to bring about an inhibition of the growth of intracellular mycobacteria, though it must be assumed that a major part of these exudate macrophages entered from the blood stream and cannot be considered cells without sufficient contact with Triton WR 1339 or its possible metabolites.

As mentioned above, the experiments described here should reveal whether peritoneal macrophages in tissue culture are a simple tool for measuring cellular immunity or acquired resistance to infections provoked by chemical substances. It could be demonstrated that unstimulated peritoneal macrophages are not a true sample of the macrophages handling the bacteria in the lungs, liver, and spleen. For establishing a relevant in vitro model, it seems necessary to infect the donor of the macrophages intraperitoneally, which brings about an influx of monocytes from the bloodstream and T-lymphocytes. Under these experimental conditions, the tissue culture system with macrophages from the peritoneal cavity can indicate the cell-mediated immunity brought about by an immunization of the host despite the fact that sufficient immune lymphocytes are not present in the culture system. In contrast to these observations, this in vitro system could not be used to measure the degree of acquired resistance caused by the prior treatment with Triton WR 1339.

LITERATURE CITED

1. Ando, M., A. M. Dannenberg, Jr., and K. Shima. 1972. Macrophage accumulation, division, maturation, and digestive, and microbicidal capacities in tuberculous lesions. II. Rate at which mononuclear cells enter and divide in primary BCG lesions and those of reinfection. J. Immunol. **109**:8–19.
2. Armstrong, J. A., and P. D. Hart. 1971. Response of cultured macrophages to mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. J. Exp. Med. **134**:713–740.
3. Berthrong, M. 1968. The macrophage-tubercle bacillus relationship and resistance to tuberculosis. Ann. N.Y. Acad. Sci. **154**:157–166.
4. Bonventre, P. E., and J. G. Imhoff. 1970. Uptake of ^{3}H-dihydro-streptomycin by macrophages in culture. Infect. Immun. 2:89–95.
5. Catanzaro, Ph.J., R. C. Graham, Jr., and C. P. Burns. 1974. Mouse peritoneal lymphocytes: general properties of normal peritoneal lymphocytes. J. Reticuloendothel. Soc. **16**:150–160.
6. Catanzaro, Ph.J., R. C. Graham, Jr., and W. R. Hogrefe. 1974. Mouse peritoneal lymphocytes: a morphologic comparison of normal and exudate peritoneal lymphocytes. J. Reticuloendothel. Soc. **16**:161–174.
7. Chang, Y. T. 1964. Long-term cultivation of mouse peritoneal macrophages. J. Natl. Cancer Inst. **32**:19–35.
8. Chang, Y. T. 1969. Suppressive activity of streptomycin on the growth of *Mycobacterium lepraemurium* in macrophage cultures. Appl. Microbiol. **17**:750–754.
9. Cornforth, J. W., P. D. Hart, G. A. Nicholls, R. J. W. Rees, and J. A. Stock. 1955. Antituberculous effects of certain surface-active polyoxyethylene ethers. Br. J. Pharmacol. **10**:73–86.
10. Cornforth, J. W., P. D. Hart, R. J. W. Rees, and J. A. Stock. 1951. Antituberculous effect of certain surface-active polyoxyethylene ethers in mice. Nature (London) **168**:150–153.
11. Dannenberg, A. M., Jr. 1968. Cellular hypersensitivity and cellular immunity in the pathogenesis of tuberculosis: specificity, systemic and local nature, and associated macrophage enzymes. Bacteriol. Rev. **32**: 85–137.
12. Dannenberg, A. M., Jr., M. Ando, O. Rojas-Espinosa, K. Shima, and T. Tsuda. 1974. Macrophage activation in tuberculous lesions, p. 222–237. *In* W. H. Wagner and H. Hahn (ed.), Activation of macrophages. Workshop Conferences Hoechst, vol. 2. Excerpta Medica, Amsterdam.
13. Dannenberg, A. M., Jr., M. Ando, and K. Shima. 1972. Macrophage accumulation, division, maturation and digestive, and microbicidal capacities in tuberculous lesions. III. The turnover of macrophages and its relation to their activation and anti-microbial immunity in primary BCG lesions and those of reinfection. J. Immunol. **109**:1109–1121.
14. Dannenberg, A. M., Jr., M. S. Burstone, P. C. Walter, and J. W. Kinsley. 1963. A histochemical study of phagocytic and enzymatic functions of rabbit mononuclear and polymorphonuclear exudate cells and alveolar macrophages. I. Survey and quantitation of enzymes, and states of cellular activation. J. Cell Biol. **17**:465–486.
15. Dannenberg, A. M., Jr., O. T. Meyer, J. R. Esterly, and T. Kambara. 1968. The local nature of immunity in tuberculosis, illustrated histochemically in dermal BCG lesions. J. Immunol. **100**:931–941.
16. Dannenberg, A. M., Jr., W. G. Roessler, O. T. Meyer, S. Chandrasekhar, and T. Kambara. 1970. Radiation, infection, and macrophage function. III. Recovery from the effects of radiation illustrated by dermal BCG lesions; resistance of pulmonary alveolar macrophages to radiation illustrated by tuberculosis produced by the airborne route. J. Reticuloendothel. Soc. **7**:91–108.
17. de Duve, C., and A. Trouet. 1973. Lysosomes and lysosomotropic drugs in host-parasite relationship, p. 153–170. *In* W. Braun and J. Ungar (ed.), Nonspecific factors influencing host resistance. S. Karger, Bassel.
18. de Duve, C., Th. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. van Hoof. 1974. Lysosomotropic

agents. Biochem. Pharmacol. 23:2495–2551.
19. van Furth, R. 1974. The effect of immunosuppressive drugs on the formation and kinetics of mononuclear phagocytes, p. 178–190. *In* W. H. Wagner and H. Hahn (ed.), Activation of macrophages. Workshop Conferences Hoechst, vol. 2. Excerpta Medica, Amsterdam.
20. van Furth, R. 1970. Origin and kinetics of monocytes and macrophages. Semin. Hematol. 7:125–141.
21. van Furth, R. 1975. Modulation of monocyte production, p. 161–174. *In* R. van Furth (ed.), Mononuclear phagocytes in immunity, infection and pathology. Blackwell Scientific Publications, Oxford.
22. van Furth, R., and Z. A. Cohn. 1968. The origin and kinetics of mononuclear phagocytes. J. Exp. Med. 128:415–435.
23. van Furth, R., and M. M. C. Diesselhoff-den Dulk. 1970. The kinetics of promonocytes and monocytes in The bone marrow. J. Exp. Med. 132:813–828.
24. van Furth, R., M. E. Fedorko, and J. G. Hirsch. 1970. Morphology and peroxidase cytochemistry of mouse promonocytes, monocytes, and macrophages. J. Exp. Med. 132:794–812.
25. Goud, Th.J. L. M., C. Schotte, and R. van Furth. 1975. Identification of the monoblast in mononuclear phagocyte colonies grown in vitro, p. 189–203. *In* R. van Furth (ed.), Mononuclear phagocytes in immunity, infection and pathology. Blackwell Scientific Publications, Oxford.
26. Hahn, H. 1974. Zelluläre antibakterielle Immunität. Dtsch. Med. Wochenschr. 99:651–656.
27. Hamilton, M. A. 1959. Monocytes from normal and immunized guinea pigs infected with virulent human tubercle bacilli.Am. Rev. Tuberc. Pulm. Dis. 79:221–237.
28. Hart, P. D. 1968. *Mycobacterium tuberculosis* in macrophages: effect of certain surfactants and other membrane-active compounds. Science 162:686–689.
29. Hart, P. D. 1974. Critical approach to the technique of assessment of antibacterial effects of activated mouse peritoneal macrophages, p. 131–137. *In* W. H. Wagner and H. Hahn (ed.), Activation of macrophages. Workshop Conferences Hoechst, vol. 2. Excerpta Medica, Amsterdam.
30. Hart, P. D., J. A. Armstrong, C. A. Brown, and P. Draper. 1972. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infect. Immun. 5:803–807.
31. Hart, P. D., A. H. Gordon, and P. Jacques. 1969. Suggested role of lysosomal lipid in the contrasting effects of Triton WR-1339 and Dextran on tuberculous infection. Nature (London) 222:672–673.
32. Hart, P. D., and S. N. Payne. 1971. Effects of non-ionic surfactants that modify experimental tuberculosis on lipase activity of macrophages. Br. J. Pharmacol. 43:190–196.
33. Kambara, T., S. Chandrasekhar, A. M. Dannenberg, Jr., and O. T. Meyer. 1970. Radiation, infection, and macrophage function. I. Effects of whole body radiation on dermal tuberculous lesions in rabbits: development, histology, and histochemistry. J. Reticuloendothel. Soc. 7:53–78.
34. Kief, H. 1957. Ueber gewebliche Reaktionen der Rattenlunge bei experimenteller Tuberkulose. Zentralbl. Allg. Pathol. Pathol. Anat. 96:40.
35. Koster, F. T., and D. D. McGregor. 1970. Rat thoracic duct lymphocyte types that participate in inflammation. Science 167:1137–1139.
36. Koster, F. T., D. D.McGregor, and G. B. Mackaness. 1971. The mediator of cellular immunity. II. Migration of immunologically committed lymphocytes into inflammatory exudates. J. Exp. Med. 133:400–409.
37. Koster, F. T., D. D. McGregor, and G. B. Mackaness. 1971. The mediator of cellular immunity. III. Lymphocyte traffic from the blood into inflammatory exudates. J. Exp. Med. 133:864–876.
38. Lefford, M. J. 1975. Transfer of adoptive immunity to tuberculosis in mice. Infect. Immun. 11:1174–1181.
39. Lefford, M. J., D. D. McGregor, and G. B. Mackaness. 1973. Properties of lymphocytes which confer adoptive immunity to tuberculosis in rats. Immunology 25:703–715.
40. Lefford, M. J., D. D. McGregor, and G. B. Mackaness. 1973. Immune response to *Mycobacterium tuberculosis* in rats. Infect. Immun. 8:182–189.
41. Lovelock, J. E., and R. J. W. Rees. 1955. Possible site and mode of action of certain lipotropic macromolecules in tuberculosis. Nature (London) 175:161–163.
42. Lurie, M. B. 1964. Resistance to tuberculosis: experimental studies in native and acquired defensive mechanisms. Harvard University Press, Cambridge.
43. McGregor, D. D., H. Hahn, and G. B. Mackaness. 1973. The mediator of cellular immunity. V. Development of cellular resistance to infection in thymectomized irradiated rats. Cell. Immunol. 6:186.
44. McGregor, D. D., F. T. Koster, and G. B. Mackaness. 1971. The mediator of cellular immunity. I. The lifespan and circulation dynamics of the immunologically committed lymphocyte. J. Exp. Med. 133:389.
45. Mackaness, G. B. 1952. The action of drugs on intracellular tubercle bacilli. J. Pathol. Bacteriol. 64:429–446.
46. Mackaness, G. B. 1954. Artificial cellular immunity against tubercle bacilli. An effect of polyoxyethylene ethers (Triton). Am. Rev. Tuberc. Pulm. Dis. 69:690–707.
47. Mackaness, G. B. 1954. The growth of tubercle bacilli in monocytes from normal and vaccinated rabbits. Am. Rev. Tuberc. Pulmon. Dis. 69:495–504.
48. Mackaness, G. B. 1956. The intracellular activation of pyrazinamide and nicotinamide. Am. Rev. Tuberc. Pulmon. Dis. 84:718–728.
49. Mackaness, G. B. 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. 120:105–120.
50. Mackaness, G. B. 1968. Editorial: The immunology of anti-tuberculous immunity. Am. Rev. Respir. Dis. 97:337–344.
51. Mackaness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med. 129:973–992.
52. Mackaness, G. B. 1971. Delayed hypersensitivity and the mechanism of cellular resistance to infection, p. 413–424. *In* B. Amos (ed.), Progress in immunology. Proc. 1st Int. Congr. Immunol. Academic Press Inc., New York.
53. Mackaness, G. B., and N. Smith. 1952. The action of isoniazid (isonicotinic acid hydrazide) on intracellular tubercle bacilli. Am. Rev. Tuberc. Pulmon. Dis. 66:125–133.
54. Mackaness, G. B., and N. Smith. 1953. The bactericidal action of isoniazid, streptomycin and terramycin on extracellular and intracellular tubercle bacilli. Am. Rev. Tuberc. Pulmon. Dis. 67:322–340.
55. Mackaness, G. B., N. Smith, and A. Q. Wells. 1954. The growth of intracellular tubercle bacilli in relation to their virulence. Am. Rev. Tuberc. Pulmon. Dis. 69:479–494.
56. Meyer, O. T., and A. M. Dannenberg, Jr. 1970. Radiation, infection, and macrophage function. II. Effect of whole body radiation on the number of pulmonary alveolar macrophages and their hydrolytic enzymes. J. Reticuloendothel. Soc. 7:79–30.
57. Myrvik, Q. N., E. S. Leake, and B. Farris. 1961. Pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. J.

Immunol. 86:128–132.
58. Myrvik, Q. N., E. S. Leake, and B. Fariss. 1962. Lysozyme content of alveolar and peritoneal macrophages from rabbit. J. Immunol. 86:133–136.
59. Myrvik, Q. N., E. S. Leake, and S. Oshima. 1962. A study of macrophages and epitheloid-like cells from granulomatous (BCG-induced) lungs of rabbits. J. Immunol. 89:745–751.
60. Nelson, D. S. 1969. Macrophages and immunity. North-Holland Publishing Co., Amsterdam.
61. North, R. J., and G. Spitalny. 1974. Inflammatory lymphocyte in cell-mediated antibacterial immunity: factors governing the accumulation of mediator T cells in peritoneal exudates. Infect. Immun. 10:489–498.
62. Patterson, R. J., and G. P. Youmans. 1970. Demonstration in tissue culture of lymphocyte-mediated immunity to tuberculosis. Infect. Immun. 1:600–603.
63. Patterson, R. J., and G. P. Youmans. 1970. Multiplication of *Mycobacterium tuberculosis* within normal and "immune" mouse macrophages cultivated with and without streptomycin. Infect. Immun. 1:30–40.
64. Pearsall, N. N., and R. S. Weiser. 1970. The macrophage. Lea and Febiger, Philadelphia.
65. Shima, K., A. M. Dannenberg, Jr., M. Ando, S. Chandrasekhar, J. A. Seluzicki, and J. I. Fabrikant. 1972. Macrophage accumulation division, maturation, and digestive, and microbicidal capacities in tuberculous lesions. I. Studies involving their incorporation of tritiated thymidine and their content of lysosomal enzymes and bacilli. Am. J. Pathol. 67:159–176.
66. Simon, H. B., and J. N. Sheagren. 1971. Cellular immunity in vitro. I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. Exp. Med. 133:1377–1389.
67. Simon, H. B., and J. N. Sheagren. 1972. Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell Immunnol. 4:163–174.
68. Solotorovsky, M., and L. S. Soderberg. 1972. Host-parasite interactions with macrophages in culture, p. 385–431. *In* A. I. Laskin and H. Lechevalier (ed.), Macrophages and cell immunity. CRC Press, Cleveland.
69. Suter, E. 1953. Multiplication of tubercle bacilli within mononuclear phagocytes in tissue culture derived from normal animals and animals vaccinated with BCG. J. Exp. Med. 97:235–000.
70. Thompson, J., and R. van Furth. 1970. The effect of glucocorticosteroids on the kinetics of mononuclear phagocytes. J. Exp. Med. 131:429–442.
71. Trouet, A. 1964. Immunisation de lapins par des lysosomes hépatiques de rats traités au Triton WR 1339. Arch. Int. Physiol. Biochim. 72:698–705.
72. Tulkens, P., and A. Trouet. 1972. Uptake and intracellular localization of streptomycin in the lysosomes of cultured fibroblasts. Arch. Int. Physiol. Biochim. 80:623–624.
73. Wagner, W. H. 1964. Experimentelle Infektionen mit Tuberkelbakterien, p. 354–430. *In* O. Eichler and A. Fahrah (ed.), Handbuch der experimentellen Pharmakologie. Springer-Verlag, Berlin.
74. Wagner, W. H. 1974. Activation of macrophages from different rodent species, p. 123–130. *In* W. H. Wagner and H. Hahn (ed.), Activation of macrophages. Workshop Conferences Hoechst, vol. 2. Excerpta Medica, Amsterdam.
75. Wagner, W. H., and G. Saar. 1954. Die quantitative Bestimmung von Tuberkelbakterien mittels Zählplatten. Arb. Paul-Ehrlich-Inst. 51:178–186.