

Multiplication of *Mycobacterium tuberculosis* Within Normal and "Immune" Mouse Macrophages Cultivated With and Without Streptomycin

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Acquired cellular immunity to infection with *Mycobacterium tuberculosis* is believed to reside in the capacity of mononuclear phagocytes of immunized animals to inhibit intracellular multiplication of the parasite. However, in macrophage tissue culture systems, it has been customary to employ streptomycin in the medium for the purpose of restricting extracellular, but not intracellular, growth of *M. tuberculosis*. In contrast, our data show that small amounts of streptomycin markedly inhibit intracellular as well as extracellular growth of *M. tuberculosis* in normal mouse peritoneal macrophages, and that the degree of this inhibition is directly proportional to the concentration of streptomycin used. In the absence of streptomycin, virulent tubercle bacilli grew as rapidly in "immune" macrophages as in normal macrophages. "Immune" macrophages, however, were slightly more resistant to destruction by the intracellularly multiplying mycobacteria. In the presence of streptomycin, however, intracellular mycobacterial growth was inhibited more in "immune" macrophages than in normal macrophages, and this effect also was directly proportional to the concentration of streptomycin used. Virulent mycobacteria grew somewhat more slowly within mouse peritoneal macrophages obtained after induction of a peritoneal exudate with glycogen than in noninduced cells. The rate of multiplication, though, was the same within normal and "immune" induced peritoneal cells except in the presence of streptomycin. As with noninduced macrophages, this drug inhibited the intracellular multiplication of virulent tubercle bacilli more effectively within "immune" induced than within normal induced cells. It would appear, therefore, that the greater inhibition of intracellular multiplication of virulent tubercle bacilli in "immune" macrophages in tissue culture noted by a number of investigators in the past may have been an artifact created by the use of streptomycin in the tissue culture medium.

The ability of mononuclear phagocytes of immunized animals to inhibit the proliferation of facultative intracellular parasites is the current definition of acquired cellular immunity. This concept is apparently supported by a considerable amount of experimental evidence. Lurie (29, 30) first demonstrated that virulent mycobacteria did not multiply in immunized rabbits. In addition, Lurie (31) found that the phagocytic cells from immunized animals usually inhibited mycobacterial proliferation to a greater extent than did normal cells when injected into the anterior chamber of the rabbit eye. This restriction of intracellular growth seemed to be solely a property of the phagocytic cell, because immune serum plus immune cells yielded results similar to those obtained with immune cells and normal serum.

A large body of subsequent evidence which appeared to support Lurie's findings was obtained by the use of phagocytic cells cultivated and infected *in vitro*, although contradictory results have been obtained by different investigators. However, the majority have reported that "immune" macrophages partially restricted the intracellular proliferation of mycobacteria (2, 26, 27, 42, 49), whereas normal macrophages permitted intracellular growth (1, 2, 26-28, 33, 47, 49).

In the majority of these studies, streptomycin has been incorporated into the tissue culture medium for the purpose of controlling extracellular growth of the microorganism used for the infection of the cells (1, 2, 26, 27, 32, 33, 47-49). In our attempt to establish the macrophage culture system as an assay for acquired cellular

immunity to tuberculosis, we noted that both normal and "immune" macrophages from mice were destroyed by virulent mycobacteria when cultured in the absence of streptomycin, but not in the presence of the antibiotic. A study of this phenomenon showed that streptomycin inhibited intracellular mycobacterial proliferation and that the effect was dependent upon the concentration of the antibiotic in the medium. Furthermore, 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.

MATERIALS AND METHODS

Mycobacterial strains. The attenuated H37Ra strain of *Mycobacterium tuberculosis* was used as the immunizing agent. The virulent H37Rv strain was used for both *in vivo* and *in vitro* infection. Both of these strains were maintained by subculture on a modified Proskauer and Beck synthetic medium (50).

Vaccination. The method used for vaccination was that previously described in detail by Youmans and Youmans (52). Briefly, male CF-1 mice were vaccinated intraperitoneally with 1.0 mg (moist weight) of a suspension of a 2-week-old pellicle culture of living H37Ra cells contained in 0.2 ml of 0.01 M phosphate buffer solution, pH 7.0.

***In vivo* challenge and measurement of immune response.** The methods used have been previously described (52). Both vaccinated and control mice were infected by the intravenous injection of 1.0 mg of a well-dispersed suspension of the virulent H37Rv strain of *M. tuberculosis*. A record was kept of the time of death of each experimental animal, and the number of 30-day survivors (S-30 mice) in the vaccinated group was compared with the number of S-30 mice among the nonvaccinated controls. The rationale for and the validity of this evaluation procedure has been thoroughly covered (52).

Preparation of H37Rv cells for macrophage infection. To eliminate appreciable numbers of clumps of bacilli and thus ensure that most of the macrophages would be infected with single acid-fast rods, the following procedure was adopted. A 2- to 3-week-old pellicle growth of H37Rv was ground in a mortar and pestle with 0.01 M phosphate buffer, pH 7.0, as a diluent (52). After allowing large clumps of bacilli to settle out by standing, the supernatant fluid was centrifuged at $200 \times g$ for 10 min in an International model CS centrifuge. The supernatant fluid was then filtered through a double layer of Celeritas filter paper. The filtrate was used for *in vitro* infection of macrophages after determining the moist weight of bacilli by centrifugation in a Hopkins vaccine centrifuge tube (52). Ziehl-Nielsen stained slides of such preparations revealed a suspension composed almost entirely of single acid-fast bacilli.

Collection and cultivation of macrophages. Chang's (7) procedure for the collection of peritoneal cells was used with certain modifications. Mice were killed

by cervical fracture, and 4.0 ml of a solution composed of 75% NCTC 109 and 25% horse serum, containing 5 units of heparin per ml of solution, was injected intraperitoneally and the abdomen was massaged gently. The skin was reflected and a small incision was made just below the tip of the xyphoid. A bent-tip sterile Pasteur pipette was used to withdraw the instilled fluid. In this manner, 3 to 3.5 ml of the fluid per mouse could be recovered. In some cases, macrophages were obtained from both normal and immune mice that had received an intraperitoneal injection of 1.0 ml of 1.0% glycogen 48 hr prior to cell collection. The peritoneal cells from 10 mice were pooled and were centrifuged at $200 \times g$ for 10 min in a sterile conical centrifuge tube. The cells were resuspended in 30 to 40 ml of sterile saline (0.85% NaCl) and re-centrifuged. This saline wash was repeated, and the final cell pellet was suspended in 15 ml of NCTC 109. White cell counts were performed by use of a hemocytometer. May-Grunwald-Giemsa stained smears revealed approximately 65% macrophages and 35% lymphocytes and polymorphonuclear cells for normal mice and 30% macrophages and 70% lymphocytes and polymorphonuclear cells for immunized mice.

For cultivation of the macrophages, acid-washed sterile cover slips (10.5×22 mm) were placed in petri dishes, and 0.2 ml of the macrophage suspension was carefully pipetted onto each cover slip. The petri dishes were incubated at 37 C in a CO₂ incubator for 1 hr to allow the cells to adhere to the cover slips. Sterile saline was added to each petri dish to remove nonadhering cells, and the cover slips were transferred to new petri dishes containing fresh culture medium (55% NCTC 109, 40% horse serum, 5% of a 1:5 dilution of bovine embryo extract). Before being infected, the cells were allowed to adjust to the culture conditions for 24 to 36 hr in a CO₂ incubator that maintained the pH of the medium between 7.2 and 7.4.

Infection of macrophages. By altering the number of infecting organisms, different levels of parasitism were attained. Usually a ratio of two to five bacilli per peritoneal cell resulted in infection of 40 to 60% of the macrophages with an average of two to three bacilli per infected cell. The desired number of virulent H37Rv cells was added to 10 ml of a 75% NCTC 109, 25% horse serum solution. The tissue culture medium was removed from each petri dish by aspiration, and the medium containing bacilli was added. The cells were allowed to phagocytize the bacilli for 1 hr in the CO₂ incubator. The infecting medium was removed by aspiration and the cells were washed two or three times with 30 to 40 ml of sterile saline to remove nonphagocytized mycobacteria. Fresh culture medium either with or without streptomycin, but always containing 50 units of both penicillin and nystatin per ml, was added to each petri dish. Preliminary studies showed that this concentration of nystatin had no effect on the growth of the virulent H37Rv in tissue culture medium. The H37Rv strain of *M. tuberculosis* was completely inhibited by 0.625 μ g of streptomycin per ml in Proskauer and Beck synthetic medium. The cell culture medium was changed at 24- or 48-hr intervals.

Measurement of mycobacterial and macrophage populations. Approximately 2 hr after infection, and at least every 5 days subsequently, three randomly selected cover slips were removed from the petri dishes, fixed in Zenker's solution for 15 min, stained for 5 min in steaming carbol-fuchsin, decolorized in acid-alcohol, stained for 45 to 50 sec in Papanicolaou hematoxylin, and blued with ammonia water. The cover slips were air-dried and mounted in Permunt for microscopic examination.

One hundred infected macrophages per cover slip were randomly examined, and the number of intracellular bacilli was recorded. The data from the three cover slips per day were averaged. All counts were made by the same investigator and were made only after the cover slips were coded.

The data were expressed in the following ways: (i) number of intracellular bacilli per 100 infected macrophages; (ii) number of intracellular bacilli per 100 macrophages of the total population; (iii) percentage of infected macrophages; and (iv) number of macrophages per 100 infected macrophages containing more than 20 intracellular bacilli and designated too numerous to count (TNTC). The number of bacilli in the TNTC macrophages was set at 30 for day 0 and 200 for days 5, 10, and 20. These numbers were experimentally determined with as much accuracy as possible and used when determining the total approximate number of intracellular mycobacteria.

To compare experiments with different levels of parasitism, the ratio of the number of intracellular bacilli per 100 macrophages of the total population at days 5, 10, and 20 to the number of intracellular bacilli at day 0 was calculated and expressed as the mean percentage increase of intracellular bacilli over day 0. The number of intracellular bacilli per 100 macrophages of the total population was believed to represent the most valid picture of the dynamic host-parasite relationship, but the same general trends prevailed regardless of the manner in which the data were expressed.

The number of macrophages per cover slip was determined in the following manner. Twenty random fields were chosen, the number of cells per field was recorded, and the number of macrophages per cover slip was calculated. The number of attached macrophages did not differ significantly at the 5% level between the three cover slips as determined by analysis of variance.

The number of extracellular bacilli in the culture medium was determined by the small inocula culture method previously described in detail by Youmans and Youmans (51).

RESULTS

Uninfected normal macrophages. Normal macrophages could be maintained in cell culture for 3 to 4 weeks. Microscopic counts of glass-adhering cells revealed 100% survival during the first 5 days in culture. By the tenth day, 11% of the macrophages were lost, a 20% loss occurred by day 15, and 34% were detached by day 20.

During the next 5 days, about 60% of the original number of macrophages had disappeared.

The morphological appearance and behavior of normal macrophages in culture were similar to the description given by Chang (7). The macrophages readily adhered to the cover slips, whereas most of the lymphocytes and polymorphonuclear leukocytes were removed by the saline wash due to their inability to stick to glass. Various shapes of macrophage nuclei were observed. Most nuclei were kidney or horseshoe shaped, but ring-type nuclei were also noted. Binucleated cells were seen frequently. Within the 1 hr permitted for attachment, many of the macrophages had developed pseudopodia, and by 24 hr all of the cells were active in this respect. The macrophages increased in size during culture and the nuclei tended to become more round or oval in appearance. Large epithelioid cells were occasionally present in the cultures.

The two criteria adopted to determine viability of the cultured macrophages were the ability to phagocytize attenuated tubercle bacilli and the morphology of the cells. Infection of the cultures at days 0, 5, 10, and 20 with a constant number of cells of the attenuated H37Ra strain of *M. tuberculosis* resulted in the same percentage of infected macrophages. The morphological appearance of pseudopodia and the ruffled cell margins exhibited by nearly all of the cells also indicated a high percentage of viable cells. In no instances were mitotic figures observed nor was there an increase in the number of cells attached to the cover slips.

Infected normal macrophages. The percentage increase in intracellular H37Rv cells within normal macrophages cultured both in the presence and absence of 5 μ g of streptomycin per ml of medium (Fig. 1) shows that without the antibiotic a mean 2,800% increase in the number of intracellular bacilli occurred by day 5 and that most of the macrophage cultures were destroyed within the next few days. Concomitantly, the percentage of surviving macrophages decreased in an exponential fashion (Fig. 2). Approximately 70% of the host cells were destroyed by day 5, but this varied depending upon the initial level of infection.

The problem created by extracellular proliferation of nonphagocytized bacilli which could have caused macrophage destruction was next examined. The number of extracellular bacilli in the culture medium was determined at 24-hr intervals, and the number of intracellular bacilli and the number of macrophages was determined at days 0 and 5 (Table 1). The bacilli required for the initiation of extracellular proliferation may have

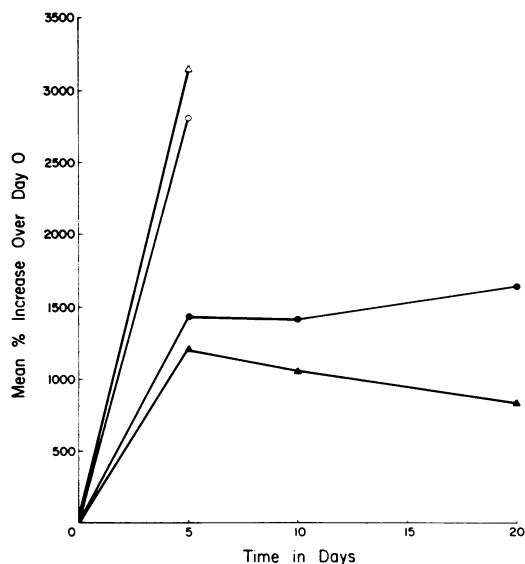


FIG. 1. Mean percentage increase of virulent *H37Rv* cells within normal and "immune" macrophages cultured with and without streptomycin. Normal macrophages with streptomycin (●), without streptomycin (○). "Immune" macrophages with streptomycin (▲), without streptomycin (△).

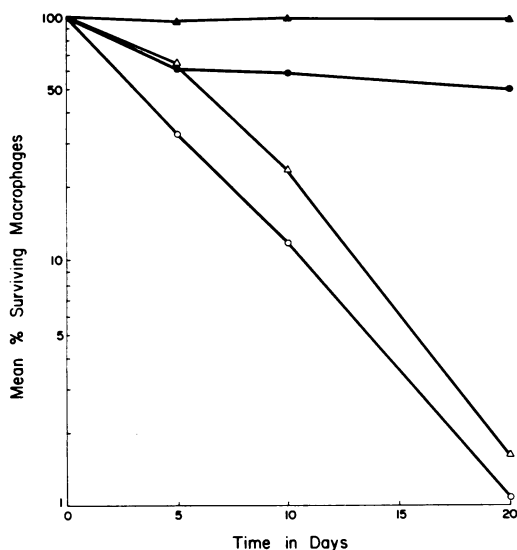


FIG. 2. Mean percentage of surviving normal and "immune" macrophages cultured with and without streptomycin after *in vitro* infection with virulent *H37Rv* cells. Normal macrophages with streptomycin (●), without streptomycin (○). "Immune" macrophages with streptomycin (▲), without streptomycin (△).

TABLE 1. Number of surviving normal macrophages after *in vitro* infection with virulent *H37Rv* cells, and number of *H37Rv* cells found in the culture medium daily

Day	No. of macrophages per cover slip	Calculated no. of macrophages lost	No. of extracellular <i>H37Rv</i> cells per cover slip
0	85,008	—	—
1	57,805 ^a	27,203 ^b	47 × 10 ⁵
2	38,254 ^a	19,551 ^b	63 × 10 ⁵
3	25,502 ^a	12,752 ^b	65 × 10 ⁵
4	17,002 ^a	8,500 ^b	63 × 10 ⁵
5	11,200	5,802 ^b	47 × 10 ⁵

^a Calculated number of surviving macrophages.

^b Calculated number of lost macrophages assuming the loss was exponential. The total number of intracellular bacilli per cover slip at day 0 was 4.7×10^5 .

come from either an intracellular or an extracellular location. Assuming that washing the macrophages after phagocytosis removed all nonphagocytized bacilli, then the predecessors of the tubercle bacilli observed in the medium at day 1 must have come from an intracellular locale. We know that the macrophages on a cover slip at day 0 contain 4.7×10^5 bacilli (Table 1). Liberation of all of these bacilli and subsequent extracellular proliferation (with a known generation time of 18 hr in our cell culture medium) would produce only 12.5×10^5 bacilli within 24 hr, a value one-fourth that actually found. It is more likely, however, that not all nonphagocytized bacilli were removed by washing. The three washes would remove, at a minimum, 99.9% of the medium containing the nonphagocytized bacilli. Infection of the macrophages was accomplished with 20×10^5 *H37Rv* cells per cover slip, of which about 5×10^5 were phagocytized. Removal of all but 0.1% (1.5×10^8) of the nonphagocytized bacilli and their extracellular multiplication would generate only 4×10^8 bacilli within 24 hr, less than 0.1% of the number actually found.

As will be discussed later, the increase in intracellular mycobacteria and the decrease in viable macrophages can be explained only by intracellular proliferation of the bacilli followed by lysis of heavily infected cells and subsequent extracellular multiplication of these liberated bacilli.

The addition to the medium of 5 μ g of streptomycin per ml altered this pattern. The number of intracellular bacilli increased about 1,400% within the first 5 days and increased slightly during the next 15 days (Fig. 1). A mean 33% reduction in the number of macrophages occurred during the

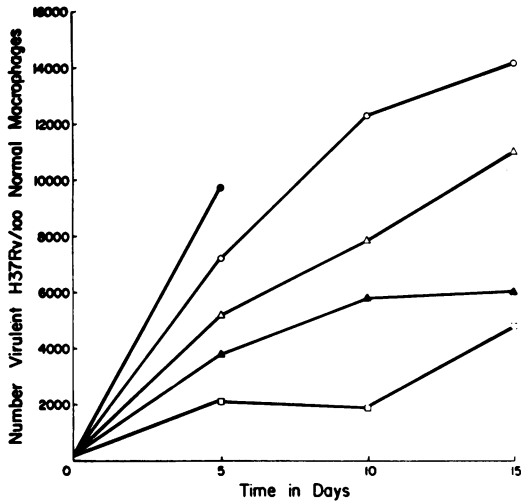


FIG. 3. Effect of various concentrations of streptomycin on the intracellular proliferation of virulent H37Rv cells within normal macrophages. Units of streptomycin per ml: 0 (●), 1.25 (○), 2.5 (△), 5.0 (▲), 10.0 (□).

first 5 days, and at day 20 only 50% of the host cells were destroyed (Fig. 2). Thus, streptomycin appeared to restrict intracellular mycobacterial multiplication and permit increased survival of the macrophages. If so, this inhibitory effect should be dose-dependent. This was confirmed by the data shown in Fig. 3.

Tables 2 and 3 record the number of individual macrophages containing from 1 to more than 20 bacilli, the percentage of infected cells, and the number of intracellular bacilli per 100 macrophages of the total population, when medium either with or without streptomycin was used. These data show the high reproducibility of infection from cover slip to cover slip. Those cells containing too many bacilli to count (TNTC) at day 0 reflect cells that have ingested some of the few clumps of bacilli known to be present in the infecting inoculum. The number of macrophages containing small numbers of bacilli decreased with time, and there was a corresponding increase in the number of heavily infected macrophages when streptomycin was absent from the medium (Table 2). This shift was less marked when the antibiotic was employed (Table 3).

Most previous investigators have used induced peritoneal exudates in their in vitro assays of cellular immunity. To determine the effect of such stimulation, glycogen-induced peritoneal macrophages were used in several experiments. It was found that induced macrophages either with or without streptomycin restricted intracellular my-

cobacterial proliferation to a greater degree than did noninduced cells (Table 4).

Uninfected "immune" macrophages. Establishment of macrophages from immunized mice in cell culture proved more difficult than establishment of macrophages from normal mice, as cells from immunized mice did not initially adhere to the cover slips as well as the normal cells. Approximately 35% of the "immune" cells adhered within 1 hr as compared with about 70% of the normal macrophages. The "immune" cells did not become actively motile until after 36 to 48 hr in culture, compared with 24 hr for the normal cells; then viability paralleled that observed with normal cells. Mitotic figures were regularly apparent in stained preparations, and in six of seven experiments an increase in the total number of cells adhering to the cover slips was noted.

Macrophages were removed from immunized mice 1, 2, 4, and 5 weeks after intraperitoneal vaccination with the attenuated strain (H37Ra) of *M. tuberculosis*, and at no time were acid-fast rods visible intracellularly. The mice used as a source of "immune" macrophages were judged to be immune by challenge of comparably vaccinated mice with the virulent H37Rv strain. Comparison of S-30 mice in the vaccinated group with the S-30 mice in the nonvaccinated group (Table 5) showed that the vaccinated animals had a marked increase in resistance to infection.

Infected "immune" macrophages. Figure 1 shows the mean percentage increase of intracellular H37Rv cells within "immune" macrophages with and without streptomycin in the medium. Without the antibiotic in the medium, the bacilli multiplied within "immune" cells, and the rate of multiplication was similar to the rate within normal cells.

Macrophage survival, however, shows a difference between the two cell types. Within the first 5 days after in vitro infection, only 30% of the "immune" macrophages were destroyed by intracellular microbial proliferation, as contrasted to the 70% reduction in the normal cells (Fig. 2). However, by day 10, the difference in survival was slight, and the death of the "immune" cells proceeded logarithmically. The initial level of infection of the "immune" cells determined the rapidity of the macrophage destruction.

The effect of streptomycin on bacterial proliferation within "immune" macrophages provided a most interesting finding. The data show that the antibiotic exerted a greater antimycobacterial growth inhibitory effect in concert with "immune" cells than when used with normal macrophages. Within the first 5 days after infection, only a mean 1,200% increase over day 0

TABLE 2. Number of normal macrophages containing 1 to more than 20 virulent H37Rv cells cultured in the absence of streptomycin^a

Time (days)	Cover slip no.	No. of macrophages containing 1 to more than 20 H37Rv cells																	TNTC	No. of H37Rv cells per 100 total cells				
		1 ^b	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17			18	19	20	
0	1	17	12	9	13	9	7	3	1	2	4	6	1	1	3	0	0	0	1	0	0	10	693	
	2	13	17	16	8	11	3	5	0	2	2	2	1	1	2	2	1	1	1	0	0	8	602	
	3	11	16	13	10	8	5	4	7	4	2	5	1	0	1	1	1	1	1	1	0	8	636	
5	Mean																					644		
	SD																						±45.99	
	1	0	4	1	3	1	1	5	3	2	0	0	1	3	3	2	2	1	0	2	0	65	13,077	
	2	1	2	3	5	3	2	1	3	4	1	2	3	0	3	3	3	0	2	3	3	56	11,413	
	3	2	4	4	7	5	3	2	3	1	3	1	4	2	0	0	0	2	2	1	1	52	10,581	
	Mean																					11,690		
	SD																						±1,270.90	

^a On each coverslip, 85 to 90% of the macrophages were infected.

^b Number of H37Rv cells per infected macrophage. TNTC = too numerous to count; for calculations, this represented 30 H37Rv cells at day 0 and 200 H37Rv cells at day 5.

TABLE 3. Number of normal macrophages containing 1 to more than 20 virulent H37Rv cells cultured in the presence of 5 µg of streptomycin per ml^a

Time (days)	Cover slip no.	No. of macrophages containing 1 to more than 20 H37Rv cells																				No. of H37Rv cells per 100 total cells	
		1 ^b	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		TNTC
0	1	18	17	14	10	5	5	6	1	3	2	2	1	3	2	1	0	0	1	0	0	9	580 672 722 658 ±72.03
	2	12	11	15	10	10	9	4	2	1	3	2	2	2	2	3	0	1	0	2	0	10	
	3	15	13	10	10	8	3	7	4	4	2	2	3	2	1	1	2	0	0	1	0	12	
	Mean																						
5	SD																						
	1	8	9	7	5	6	4	5	2	0	3	2	2	6	2	5	2	2	1	3	2	24	5,250 4,842 5,156 5,082 ±213.65
	2	4	9	6	11	8	4	5	1	6	3	4	2	2	5	4	0	1	1	0	2	22	
	3	9	13	8	7	4	4	5	5	3	0	3	3	2	0	1	3	1	2	1	2	24	
Mean																							
10	SD																						
	1	5	11	8	10	5	4	4	4	3	4	5	2	4	3	1	0	1	1	1	1	20	4,315 5,845 4,097 4,752 ±951.96
	2	5	5	7	2	6	3	6	7	4	3	3	3	2	1	3	1	1	4	1	1	28	
	3	11	10	9	6	3	3	8	6	4	2	1	5	2	1	3	2	1	1	2	1	19	
Mean																							
20	SD																						
	1	4	7	8	7	8	5	6	4	1	4	1	1	2	4	3	3	0	2	3	0	27	5,818 4,817 6,489 5,708 ±841.41
	2	8	6	8	6	4	10	10	3	4	5	4	2	1	3	0	1	0	1	1	1	22	
	3	5	3	6	6	7	5	3	4	6	3	4	1	2	1	4	1	3	3	2	2	30	
Mean																							
	SD																						

^a On each cover slip, 77 to 92% of the macrophages were infected.

^b Number of H37Rv cells per infected macrophage. TNTC = too numerous to count; for calculations, this represented 30 H37Rv cells at day 0 and 200 H37Rv cells at days 5, 10, and 20.

TABLE 4. Mean percentage increase of H37Rv cells within induced and noninduced peritoneal macrophages obtained from normal and vaccinated mice, and cultured with and without streptomycin

Streptomycin concn (µg/ml)	Day	Normal macrophages		"Immune" macrophages	
		Induced	Non-induced	Induced	Non-induced
5	0	—	—	—	—
	5	1,065	1,555	762	1,420
	10	921	1,648	387	1,385
	20	1,138	1,886	628	870
0	0	—	—	—	—
	5	2,117	3,189	2,232	3,727

TABLE 5. Immune response of mice vaccinated with 1 mg (moist weight) of living H37Ra cells

Expt	No. of mice	Percentage of S-30 mice ^a
1	28	82.1
2	30	90.0
3	27	— ^b
4	30	70.0
5	30	96.7
6	30	96.7
7	26	88.5
8	29	44.8

^a S-30 = percentage of mice which survived > 30 days. The S-30 of nonvaccinated mice ranged between 7 and 23%, except in experiments 1 and 2, where the values were 27 and 30%, respectively.

^b Experimental failure for technical reasons.

occurred in the number of intracellular bacilli. There was a slight decrease over the following 15 days (Fig. 1). Figure 4 demonstrates that the growth-inhibiting property of streptomycin also was dose-dependent when "immune" cells were used. Accompanying this restriction of the proliferation of the bacilli, there was an increased survival of the macrophages. As Fig. 2 shows, nearly 100% macrophage survival was achieved. In fact, in five of seven experiments with streptomycin, the number of "immune" macrophages increased.

Macrophages from immunized mice stimulated with glycogen 48 hr before collection were used in several experiments. Table 4 compares the intracellular multiplication of H37Rv in induced and noninduced "immune" macrophages. It is apparent that induced "immune" macrophages also restricted the intracellular proliferation of the

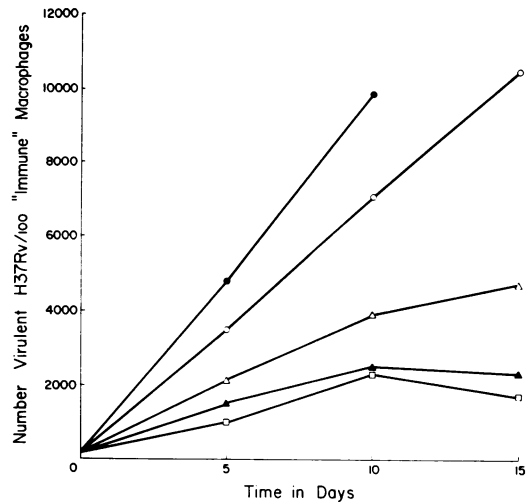


FIG. 4. Effect of various concentrations of streptomycin on the intracellular proliferation of virulent H37Rv cells within "immune" macrophages. Units of streptomycin per ml; 0 (●), 1.25 (○), 2.5 (△), 5.0 (▲), 10.0 (□).

mycobacteria whether or not streptomycin was present in the medium.

DISCUSSION

The interpretation of results of other investigators obtained from experiments in which macrophage cultures were used as an assay for cellular immunity has been complicated because the number of surviving macrophages was not counted at intervals after in vitro infection (1, 2, 5, 18, 21, 25, 28, 32, 33, 35, 36, 41, 43, 47-49). In contrast, we have determined not only the number of intracellular mycobacteria, but also the number of surviving macrophages, and, in some cases, the number of extracellular bacilli. We have obtained, therefore, a more complete picture of the dynamics and complexity of this in vitro tubercle bacillus-macrophage relationship.

Early attempts by Mackaness (32) and Suter (47) to define the parameters of this in vitro system revealed that normal macrophages infected with virulent strains of mycobacteria were destroyed 3 to 7 days after infection. Suter (47) stated that virulent tubercle bacilli (H37Rv) and attenuated strains (R1Rv, BCG-Phipps, and BCG-Tice) not only multiplied abundantly within normal macrophages, but also multiplied extracellularly. This implied that the extracellular multiplication was the major cause of the destruction of the macrophages. Streptomycin was incorporated into the medium in small amounts, sup-

posedly to restrict extracellular, but not intracellular, bacillary multiplication. When experiments were attempted to determine whether the antibiotic had any effect on intracellular bacterial growth, certain necessary parameters such as the number of surviving macrophages, the number of extracellular bacilli, the use of virulent mycobacteria, and the use of "immune" as well as normal macrophages were not evaluated.

We tried to determine the extent of the problem created by extracellular multiplication of non-phagocytized bacilli by thoroughly washing the macrophage cultures after phagocytosis and culturing the medium at 24-hr intervals to determine the number of extracellular bacilli (Table 1).

Three hypotheses can be offered to explain the number of extracellular bacilli found daily. The first states that the extracellular mycobacteria can be accounted for *only* by extracellular proliferation, but the calculations previously made show that this is not possible.

The second hypothesis postulates *only* intracellular proliferation of mycobacteria, followed by lysis of heavily infected macrophages and liberation of the intracellular bacilli into the medium with *no* subsequent proliferation outside the macrophages. Calculations based on this hypothesis, however, are meaningless owing to the fact that the bacilli do multiply in the culture medium.

The third, and most likely, hypothesis is a combination of the first two. In this hypothesis, any extracellular bacilli remaining after the cells were washed would multiply extracellularly, but would constitute only a small fraction of the total number of extracellular bacilli observed daily. The major contribution to the extracellular bacillary population would be made by the progeny of those bacilli liberated by lysis of heavily infected macrophages; this lysis would be a consequence of intracellular bacterial proliferation.

The validity of this explanation was shown by our findings that intracellular mycobacterial proliferation was exponential, with resulting macrophage loss also being exponential (Fig. 2). In addition, the greater the infecting dose, the more rapidly the macrophages were destroyed. This confirms the work of others (32, 47). Toxicity of the mycobacterial cells appeared to play little or no role as a cause of macrophage death during the first 5 days, because heavily infected cells were viable and appeared normal.

An intracellular mycobacterial generation time of between 8 and 10 hr is necessary to explain this third hypothesis. Generation times of about 24 hr for virulent H37Rv in the log phase of growth in the lungs of normal mice have been reported (37, 44). If the macrophage is considered a favorable

environment for the growth of this facultative intracellular parasite, an 8- to 10-hr generation time within the macrophage *in vitro* may not be unrealistic (6, 17). Furthermore, we have consistently obtained a generation time of 8 to 10 hr with this organism in modified Proskauer and Beck synthetic medium supplemented with 5% horse serum (*unpublished observations*).

With the assurance that death of normal macrophages and intracellular bacillary increase, without streptomycin in the medium, were due to intracellular proliferation of the parasite, experiments were performed to determine the validity of the system as an *in vitro* correlate of acquired cellular immunity. Virulent mycobacteria multiplied as rapidly in "immune" macrophages as within normal cells in the absence of streptomycin (Fig. 1). Kochan and Smith (28) also noted that macrophages from normal and BCG-immunized guinea pigs allowed equal rates of BCG proliferation intracellularly in the absence of the antibiotic.

The only difference observed between normal and "immune" macrophages in the absence of streptomycin was macrophage survival (Fig. 2). The logarithmic loss of "immune" cells was delayed until about day 5. This supports the conclusion of Fong and co-workers (19, 20) that "immune" macrophages are more resistant to the degenerative effects of intracellularly multiplying virulent mycobacteria than are normal cells. We concur in that, although virulent mycobacteria proliferate within "immune" cells as well as in normal cells, "immune" macrophages can tolerate a greater number of intracellular bacilli before succumbing to death and lysis. In this connection, Patnode and Hudgins (40) have shown that leukocytes from guinea pigs vaccinated with BCG are more resistant to sonic disruption.

Two far-reaching observations were noted when streptomycin was incorporated into the culture medium. First, small concentrations of the antibiotic can cause inhibition of intracellular microbial multiplication. Recently, Bonventre (4) reported that the uptake of the antibiotic by mouse peritoneal macrophages was delayed 2 to 4 hr, but a linear increase followed for several days until a plateau was reached. No uptake was observed at 4 C. Chang (8) also has reported that streptomycin in small concentrations inhibits proliferation of *M. lepraemurium* in mouse peritoneal macrophages. He ruled out extracellular growth and rephagocytosis of the organism as the major contributor to the increase in intracellular bacilli, as *M. lepraemurium* is an obligate intracellular parasite. Numerous other investigators have reported that macrophages (13-15, 21, 36, 38, 39) and other cells (45, 46) are permeable to strepto-

mycin and can restrict intracellular microbial multiplication.

Second, our data show that streptomycin exerts a greater growth-inhibitory effect when used with cells from immunized mice than when employed with normal cells. Kochan and Smith (28) also noted that the antibiotic inhibited the intracellular multiplication of virulent tubercle bacilli within cells from immunized guinea pigs, but did not inhibit growth of the organism within normal macrophages. Therefore, in other investigations of the inhibitory effect of "immune" macrophages on intracellular microbial proliferation (1, 2, 5, 18, 25-27, 35, 41-43, 49), it is likely that the effect of streptomycin was actually measured.

Immunization of animals with living attenuated strains of facultative intracellular parasites to induce acquired cellular immunity results in the "activation" of cells of the reticuloendothelial system (3, 11, 16, 24, 53). The use of irritants to stimulate peritoneal exudates also results in the "activation" of macrophages (10, 12). These "activated" cells are metabolically more active than normal cells (12), contain increased amounts of hydrolytic enzymes (9, 10, 24), and have increased physiological functions such as phagocytosis (12, 16, 23) and pinocytosis (3). Perhaps then, the "immune" macrophages *in vitro* may be more permeable to streptomycin and allow a more rapid uptake of the antibiotic or a greater antibiotic concentration within them, or both, thus bringing about the differential growth-inhibitory effect. Evidence that "activation" increases various physiological functions of the macrophage can be inferred from the use of glycogen-stimulated cells (Table 4). Glycogen stimulation caused approximately equal increases in the mycobactericidal (or -static) properties of both normal and "immune" macrophages in the absence of streptomycin (about 35% reduction in the number of intracellular bacilli). However, in the presence of the antibiotic, "immune" induced cells inhibited intracellular mycobacterial growth about 50% compared with only a 30% reduction of H37Rv within normal induced cells.

The data presented in this paper raise many important questions. Obviously, the most important relates to the nature of acquired cellular resistance to facultative intracellular parasites. *In vitro* studies similar to those we employed have been cited as confirmatory evidence of the concept that cellular immunity resides in the ability of "immune" macrophages to suppress the intracellular proliferation of facultative intracellular microbes. However, our data cast serious doubts on the validity of this hypothesis. What then is the mechanism of acquired cellular immunity? It

appears that the macrophage is not solely involved. Recently, Mackaness (34) reported that spleen cells from listeria-immunized animals can passively transfer cellular immunity to this organism *in vivo*. Also, the administration of anti-lymphocyte serum to immunized animals renders them more susceptible to infection with mycobacteria (22). Preliminary experiments in our laboratory indicate that spleen cells from H37Rv-vaccinated mice can bring about inhibition of intracellular proliferation of H37Rv within macrophages *in vitro*. In these experiments, streptomycin was not incorporated in the culture medium. Details of these experiments will be reported in a subsequent publication.

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