INHIBITION OF *SALMONELLA ENTERITIDIS* INGESTED IN MONONUCLEAR PHAGOCYTES FROM LIVER AND SUBCUTANEOUS TISSUE OF MICE IMMUNIZED WITH LIVE VACCINE

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SATO, ICHIEI (School of Medicine, Gunma University, Maebashi, Japan), TOKUMITSU TANAKA, KAZUKO SAITO, AND SUSUMU MITSUHASHI. Inhibition of *Salmonella enteritidis* ingested in mononuclear phagocytes from liver and subcutaneous tissue of mice immunized with live vaccine. J. Bacteriol. **83**:1306–1312. 1962.—In our earlier studies it was shown that mice hyperimmunized with live vaccine of *Salmonella enteritidis* resisted intravenous challenge with 1,000 MLD of virulent strain 116–54 of *S. enteritidis*. Survivors of this challenge completely resisted additional intravenous challenge with 10,000 MLD of the same organism. Mononuclear phagocytes obtained from the abdominal cavity of mice immunized with live vaccine of *S. enteritidis* inhibited intracellular multiplication of virulent strain 116–54, regardless of the presence of antibody in the cell-culture medium. In the present study, mononuclear phagocytes were obtained in a nearly pure state from liver or subcutaneous tissue of mice and were maintained in vitro in good condition. These cells also resisted cellular degeneration caused by intracellular existence of virulent strain 116–54, regardless of the presence of antibody in the cell-culture medium. In contrast, cells obtained from normal mice or mice immunized with dead vaccine were subject to degeneration.

In a previous paper (Mitsuhashi, Sato, and Tanaka, 1961), concerning in vitro infection with *Salmonella enteritidis* of mononuclear phagocytes derived from the abdominal cavity of mice, it was shown that the intracellular multiplication of virulent strain 116–54 was rapid, phagocytes were destroyed during 3 days of incubation, and the virulence of *S. enteritidis* for this host was recognized at the cellular level in vitro. Mononuclear phagocytes obtained from the abdominal cavity of mice immunized with live vaccine inhibited intracellular multiplication of virulent strain 116–54, regardless of the presence of antibody in the medium, whereas the cells of mice immunized with dead vaccine did not. In this paper, results of studies of certain immune effects on the growth of *S. enteritidis* in mononuclear phagocytes obtained from liver and subcutaneous tissue of mice are presented.

MATERIALS AND METHODS

The experimental animals, immunization procedure, and microorganisms used were described in a previous paper (Mitsuhashi et al., 1961).

Mononuclear phagocytes. Mononuclear phagocytes were obtained from liver or subcutaneous tissue of normal and immune mice. Phagocytes from subcutaneous tissue were obtained by the method of Akasaki (1958), and those from liver were obtained by a method devised in our laboratory. Mice were anesthetized by intraperitoneal injection of 0.1 ml of pentobarbital sodium (0.01 mg/ml in saline). After sterilization of the abdominal skin with Hyamine-T solution, a laparotomy was done through an upper mid-line incision.

A quadrate lobe and a left lobe of the liver were exposed alternately. Through a small incision made on the surface of the liver, a small cover glass (3 by 4 mm) was inserted into the tissue along the major axis of the liver. Hemorrhage was immediately controlled with sterilized cotton gauze. The peritoneum was closed with silk thread. Then two small cover glasses were placed between the abdominal muscular wall and skin on both sides. The skin was closed with silk thread. Each mouse received 500 units of penicillin and 500 μg of streptomycin. The mice were killed by heart puncture 5 to 7 days after the operation, and cover glasses to which phagocytes adhered were drawn from the liver or subcutaneous tissue.

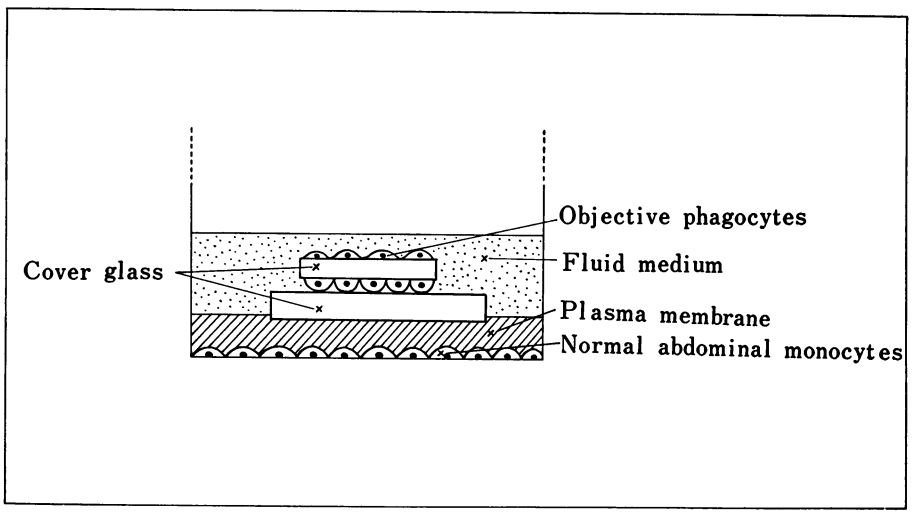

FIG. 1. *Scheme of tissue culture*

TABLE 1. *Percentages of the different types of phagocytes obtained at various times*

Cells	Days after insertion	Type of cells*			
		Pm	Mn	Pn	PnG
		%	%	%	%
Liver	1	93	7	0	0
	2	46	54	0	0
	3	3	94	1	2
	5	2	96	1	1
	7	1.6	97	0.7	0.7
	14	0	20	24	56
Subcutaneous tissue	1	75	25	0	0
	2	53	47	0	0
	3	19	78	2	1
	5	11	87	1.3	0.7
	7	3	93	3	1
	14	0	19	21	60

* Pm: polymorphonuclear leukocyte; Pn: polynuclear cell; Mn: mononuclear cell; PnG: polynuclear giant cell.

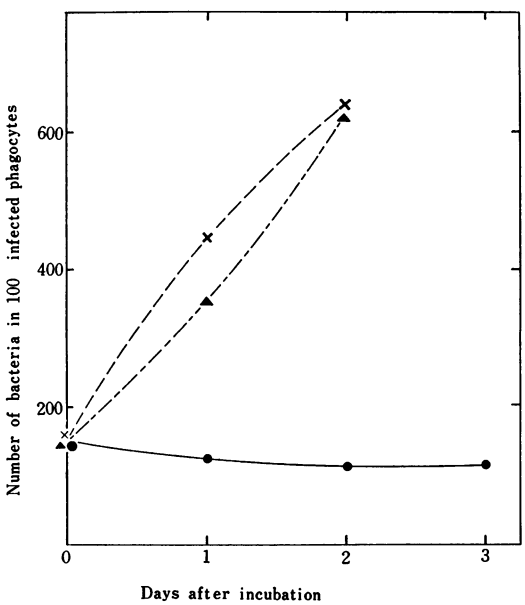

FIG. 2. *Intracellular growth of virulent strain 116–54 of Salmonella enteritidis in the phagocytes obtained from mouse liver.* ×, *phagocytes obtained from the liver of normal mice;* ▲, *phagocytes obtained from the liver of mice immunized with dead vaccine;* ●, *phagocytes obtained from the liver of mice immunized with live vaccine.*

The cells thus obtained were able to phagocytize bacteria. Six to eight pieces of cover glass obtained from one tissue were placed in a small petri dish (3 cm diam) which contained 2 ml of a medium consisting of 30% horse serum and 5% normal mouse serum.

A 10^{-2} mg/ml bacterial suspension in Hanks' solution was prepared from a fresh culture on agar, and 0.1 ml was added to a petri dish containing cover glasses to which cells adhered. The petri dish was incubated at 37 C to further phagocytosis, and after 60 min each small cover glass was taken out and washed with Hanks' solution to remove bacteria not ingested by the cells. Then the small cover glass to which infected

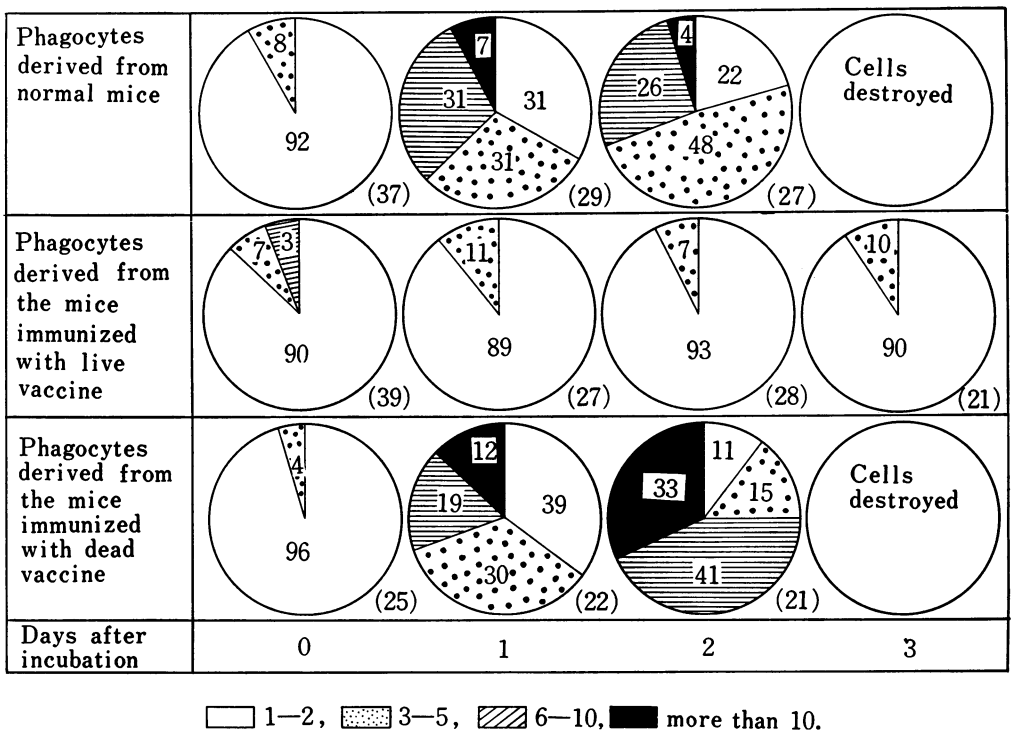

FIG. 3. *Intracellular multiplication of virulent strain 116-54 of Salmonella enteritidis in the phagocytes obtained from mouse liver. Monocytes are classified into four groups by the number of intracellular bacteria: 1-2, 3-5, 6-10, and more than 10. Figures in parentheses in each column indicate the phagocytic index.*

phagocytes adhered was incubated in a tissue-culture bottle in which abdominal monocytes had been cultivated.

Tissue culture. The abdominal mononuclear phagocytes were obtained from a normal mouse by the method described previously (Mitsuhashi et al., 1961). They were cultivated in tissue-culture bottles for 24 hr prior to the incubation of phagocytes obtained from the liver or subcutaneous tissue. The abdominal monocytes grew in a monolayer, and were covered with a plasma film (Fig. 1). The film consisted of 50% chicken plasma, 40% Hanks' solution, and 10% chick-embryo extract.

Cells were incubated at 37 C for 7 days; the culture medium was exchanged every other day. After incubation for 5 days, the cover glass to which objective phagocytes adhered was transferred into other fresh culture bottles previously prepared as described above.

A cover glass was taken from the culture bottle every day, dried in air, fixed in methanol, and stained in Giemsa solution. The number of infected phagocytes and intracellular bacteria in phagocytes was determined microscopically. The phagocytic index was defined as: the number of infected phagocytes divided by the total number of phagocytes examined times 100.

RESULTS

Effect of time on the number and types of cells adhering to the cover glass. The cover glasses were removed from mice 1, 2, 3, 5, 7, and 14 days after operation. The cells adhering to a cover glass were morphologically classified into four groups: mononuclear cells, polynuclear cells, polymorphonuclear leukocytes, and polynuclear giant cells.

The total number of cells adhering to the cover glass increased markedly 2 days after operation, and reached a maximum at 5 days. About 90% of the cells were mononuclear phagocytes 5 to 7 days after insertion of cover glasses (Table 1). The results obtained from immunized mice were almost the same as with normal mice. Cover

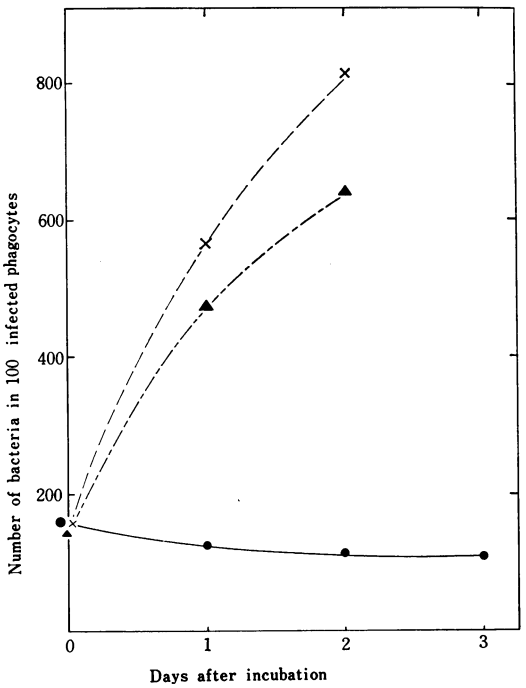

FIG. 4. *Intracellular multiplication of virulent strain 116–54 of Salmonella enteritidis in the phagocytes obtained from the subcutaneous tissue of mouse.* ×, *phagocytes obtained from the subcutaneous tissue of normal mice;* ▲, *phagocytes obtained from the subcutaneous tissue of mice immunized with dead vaccine;* ●, *phagocytes obtained from the subcutaneous tissue of mice immunized with live vaccine.*

glasses were taken out of mice 5 days after insertion, and the cells adhering to the cover glass were used for in vitro infection.

Intracellular growth of virulent strain 116–54 in mononuclear phagocytes obtained from the liver of normal or immunized mice. Of the cells obtained from mouse liver by the method described, 90 to 100% were able to phagocytize bacteria. In each experiment, the ratio of bacilli to mononuclear phagocytes was changed to produce a phagocytic index of 30 to 40%, with about 90% of the infected phagocytes ingesting one or two bacteria.

A ratio of approximately four bacilli to one mononuclear phagocyte was used. Phagocytes derived from the liver of mice immunized with dead vaccine showed a small degree of inhibition of the intracellular growth of bacteria (Fig. 2), but these phagocytes, as well as the cells of normal mice, were destroyed after 2 days of incubation (Fig. 6a, b, c).

As observed in an earlier study (Mitsuhashi, Hashimoto, and Kawakami, 1960), cells derived from mice receiving live vaccine did not allow the intracellular growth of virulent strain 116–54 during incubation, regardless of the presence of antibody in the cell-culture medium. Moreover, the bacteria within phagocytes became small in size and coccoid in shape after 2 days of incubation (Fig. 6d). Even after 7 days of incubation, the phagocytes did not show any sign of cell damage (Fig. 6e).

More detailed aspects of intracellular multiplication of virulent strain 116–54 in mononuclear phagocytes are shown in Fig. 3. The cells derived from the liver of mice immunized with live vaccine inhibited the intracellular multiplication of bacteria. But in cells of normal mice or mice immunized with dead vaccine, the number of bacteria increased and the cells were destroyed after 2 days of incubation.

Growth of virulent strain 116–54 in mononuclear phagocytes obtained from subcutaneous tissue. The behavior of bacteria in phagocytes derived from subcutaneous tissue was similar to that which occurred in phagocytes derived from the liver. The number of bacteria ingested into mononuclear phagocytes obtained from the subcutaneous tissue of normal mice or mice immunized with dead vaccine increased in the cells and reached a maximum after 2 days of incubation. In addition, the phagocytes were destroyed by intracellular multiplication of bacteria (Fig. 6f, g).

In contrast, mononuclear phagocytes obtained from subcutaneous tissue of mice immunized with live vaccine inhibited intracellular multiplication, and the number of bacteria decreased gradually (Fig. 4). As with phagocytes from the liver of mice immunized with live vaccine, the ingested bacteria became small in size and coccoid in shape (Fig. 6h).

More detailed aspects of intracellular multiplication of virulent strain 116-54 in mononuclear phagocytes are shown in Fig. 5. Cells derived from mice immunized with live vaccine inhibited intracellular multiplication of bacteria regardless of the presence of antibody in the culture media. But in the case of the cells of normal mice or mice

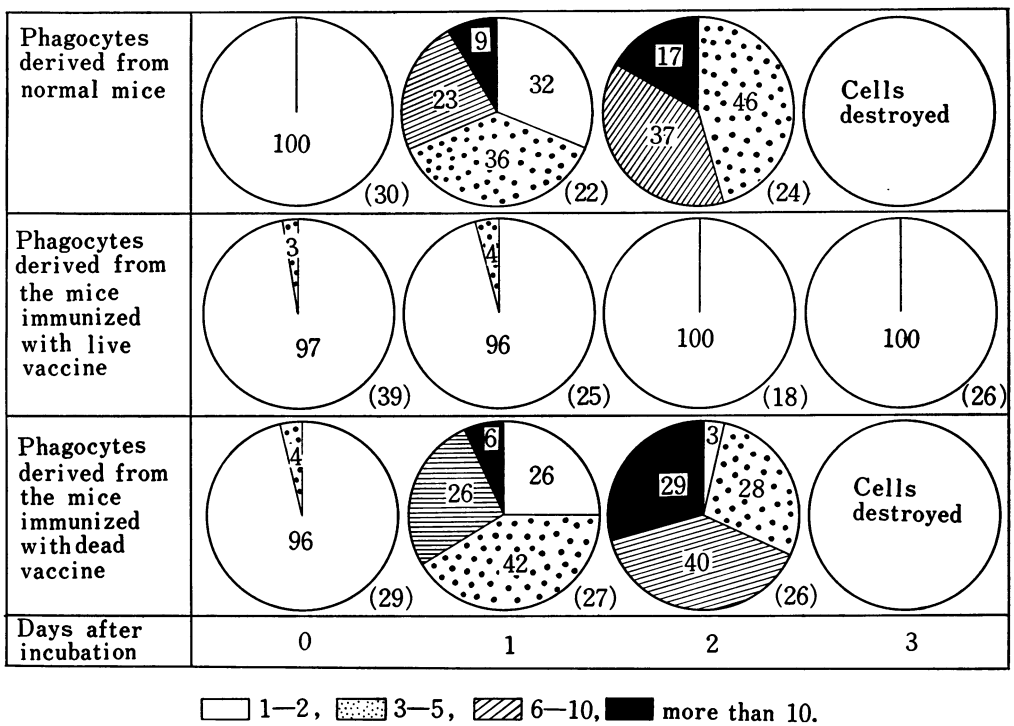

FIG. 5. *Intracellular multiplication of virulent strain 116-54 of Salmonella enteritidis in the phagocytes obtained from the subcutaneous tissue of mouse. For explanation, see Fig. 3.*

immunized with dead vaccine, bacteria increased intracellularly, and the cells were destroyed during the course of incubation.

DISCUSSION

A primary sublethal infection with certain viral and bacterial agents provokes powerful resistance to further infection by the same pathogen, and this has been found to hold for infections of mice with *S. enteritidis* (Kobayashi and Ushiba, 1951; Mitsuhashi et al., 1958). According to a recent detailed study of antilethal resistance of mice to infection with *S. enteritidis*, mice superimmunized with live vaccine resisted intravenous injection with 1,000 MLD of a virulent strain. The survivors of this challenge then completely resisted intravenous challenge with 10,000 MLD of the same organism (Mitsuhashi et al., 1960).

Antilethal resistance of mice immunized with live vaccine against infection with *S. enteritidis* lasted, so far as determined, for 16 months after

FIG. 6. (a) *Virulent strain 116-54 of Salmonella enteritidis ingested into the mononuclear phagocytes obtained from normal mouse liver. Immediately after incubation (magnification, 1,000 ×).* (b) *Intracellular multiplication of virulent strain 116-54 in phagocytes obtained from normal mouse liver; 2 days after incubation (1,000 ×).* (c) *Intracellular multiplication of virulent strain 116-54 in phagocytes obtained from the liver of mice immunized with dead vaccine; 2 days after incubation (1,000 ×).* (d) *Inhibition of intracellular multiplication of virulent strain 116-54 in the phagocytes of liver obtained from mice immunized with live vaccine; 2 days after incubation (1,000 ×).* (e) *Same as* d; *7 days after incubation (200 ×).* (f) *Intracellular multiplication of virulent strain 116-54 in the phagocytes obtained from the subcutaneous tissue of normal mice; 2 days after incubation (1,000 ×).* (g) *Intracellular multiplication of virulent strain 116-54 in phagocytes obtained from the subcutaneous tissue of mice immunized with dead vaccine; 2 days after incubation (1,000 ×).* (h) *Inhibition of intracellular multiplication of virulent strain 116-54 in phagocytes obtained from the subcutaneous tissue of mice immunized with live vaccine; 2 days after incubation (1,000 ×)*

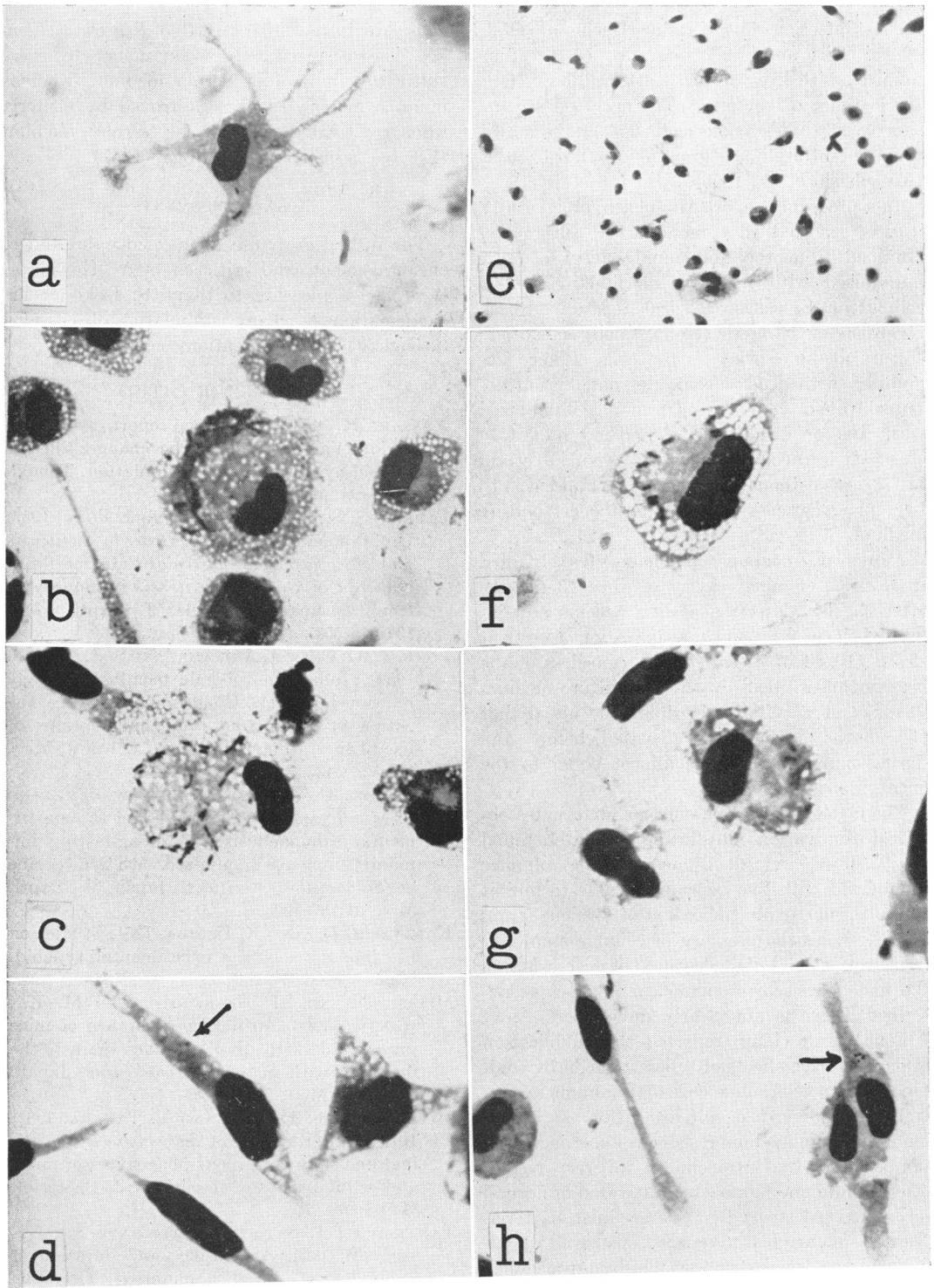

FIG. 6

the last infection of live vaccine (Hashimoto et al., 1961). This antilethal resistance of mice immunized with live vaccine is attributable mainly to clearance of bacteria from the blood stream, shortly after intravenous challenge, and to inhibition of intracellular growth of the bacteria (Mitsuhashi et al., 1960).

In a preceding paper (Mitsuhashi et al., 1961), it was reported that mononuclear phagocytes obtained from the abdominal cavity of mice immunized with live vaccine inhibited intracellular multiplication of virulent strain 116–54, regardless of the presence of antibody in the cell-culture medium, whereas the cells of mice immunized with dead vaccine did not. Serum obtained from normal mice or from mice immunized with live or dead vaccine had no inhibitory effect on intracellular growth of virulent strain 116–54, even though the serum was added both to a phagocytic system and to the cell-culture medium.

Moreover, mononuclear phagocytes obtained from the abdominal cavity of mice 13 months after the last injection of live vaccine still inhibited the intracellular multiplication of virulent strain 116–54 of *S. enteritidis*, regardless of the presence of antibody in the cell-culture medium (Tanaka et al., 1961). It should be noted that 16 months, including the period before and during immunization, are almost equal to the entire life of a mouse.

The fact that the mononuclear phagocytes obtained from various sites in the body (abdominal cavity, liver, and subcutaneous tissue) of mice immunized with live vaccine are able to inhibit the intracellular multiplication of virulent strain 116–54 shows the presence of cellular immunity in the reticuloendothelial system of mice, whereas the histogenesis of mononuclear phagocytes described above is not clearly understood.

Fong et al. (1959) reported the modification of degenerative effects of tubercle bacilli by combined action of immune cells and immune serum. Immune monocytes cultivated for 48 hr in immune serum medium resisted cellular degeneration caused by intracellular tubercle bacilli, whereas immune monocytes cultivated in normal serum were destroyed by intracellular bacteria. Normal monocytes were also destroyed in an immune or normal serum system by intracellular tubercle bacilli.

Immune monocytes obtained from the abdominal cavity of rabbits immunized with live vaccine showed resistance to cellular destruction caused by intracellular growth of *Brucella melitensis*. This occurred only when monocytes were cultivated in immune serum medium, whereas immune monocytes were destroyed by bacteria when cultivated in normal serum medium (Elberg, Schneider, and Fong, 1957).

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