tance of cells (HeLa) in culture to the cytotoxic action of influenza virus after exposure to subtoxic quantities of virus. It appears that it will be necessary to follow events in the actual cells involved in these toxicity reactions during development of host resistance in order to localize the site of altered susceptibility.

Summary. Sublethal quantities of homologous and heterologous influenza virus, mumps virus, and Newcastle disease virus injected intravenously in mice produced a state of resistance to the toxic effects of large quantities of intravenously injected PR-8 influenza A virus. Virus heated sufficiently to destroy infectivity was less effective in inducing resistance than was fully active virus; destruction of hemagglutinin caused complete loss of resistance inducing activity. Injection of sublethal doses of virus was followed by a lag period, which varied with the virus injected, before resistance became demonstrable, and resistance waned after 3-4 days. No increase in resistance to influenza toxicity was demonstrable after pre-challenge injection of feline pneumonitis virus, *R. prowazeki*, or typhoid vaccine. A preparation of receptor destroying enzyme (RDE) was effective in inducing increased resistance but its effect could not be attributed to its capacity for destruction of mucoprotein receptors since heat destruction of this activity did not completely destroy its resistance inducing effect.

1. Bennett, I. L., Jr., Wagner, R. R., and Le Quire, V. S., *J. Exp. Med.*, 1949, v90, 335.

2. Wagner, R. R., and Bennett, I. L., Jr., *ibid.*, 1950, v91, 135.

3. Wagner, R. R., *Brit. J. Exp. Path.*, 1952, v33, 157.

4. Fong, J., Louie, R., and Ching, C., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 246.

5. Groupé, V., Herrmann, E. C., Jr., and Dougherty, R. M., *ibid.*, 1954, v87, 636.

6. Henle, W., and Henle, G., *J. Exp. Med.*, 1946, v84, 639.

7. Ada, G. L., and French, E. L., *Aust. J. Sc.*, 1950, v13, 82.

8. Burnet, F. M., and Stone, J. D., *Aust. J. Exp. Biol. M. Sc.*, 1947, v25, 227.

9. Schlesinger, R. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 541.

10. Wagner, R. R., *ibid.*, 1955, v90, 214.

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Intracellular Multiplication of *Brucella abortus* in Normal and Immune Mononuclear Phagocytes.* (22860)

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Importance of mononuclear phagocytes in pathogenesis of brucellosis has been suspected by many, but the role of these cells in resistance to brucella infection has not been investigated. The intimate relationships of mononuclear phagocytic cells and brucella can be studied *in vivo* only with great difficulty. The ease with which fairly pure cultures of guinea pig mononuclear phagocytes can be obtained and maintained *in vitro* for reasonable periods of time offered a means of studying this problem. The technics used in this work allowed the numbers of both phagocytic cells and viable brucellae to be controlled with a fair degree of accuracy. Some of the difficulties inherent in working with varying ratios of bacteria and tissue cells are thus eliminated.

Materials and methods. Tissue culture technics were similar to those described by Leighton and Hanks(1). Peritoneal exudates,

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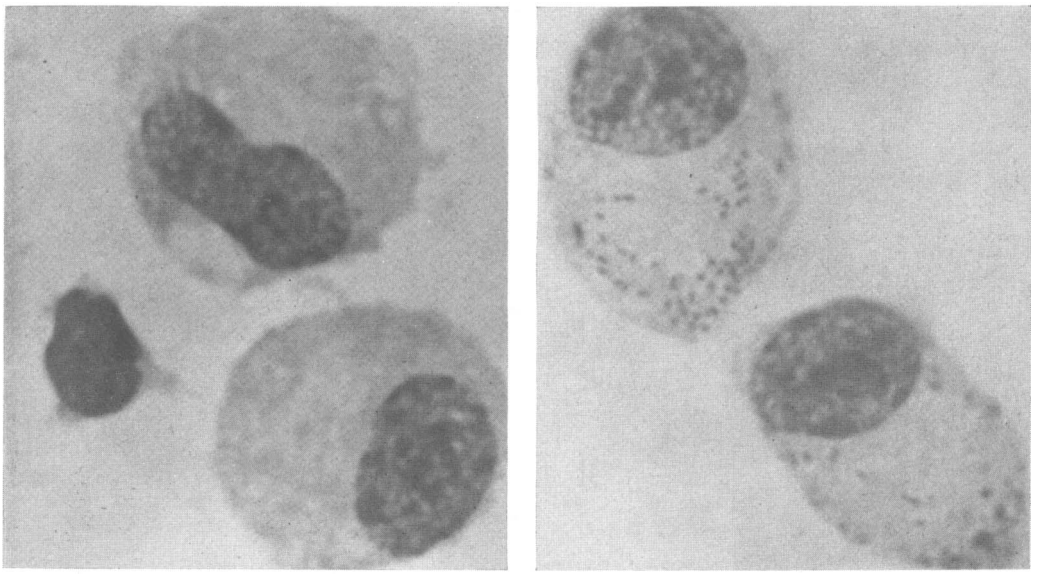

a b

FIG. 1. (a) Normal monocytes from 40 hr cultures containing 10 μg streptomycin/ml but no brucellae. (b) Normal monocytes showing intracellular brucella from 40 hr brucella containing cultures in which supernatant was replaced with sterile cell-free streptomycin containing medium after 2 hr preliminary incubation. May-Grunwald-Giemsa stain. × 2500.

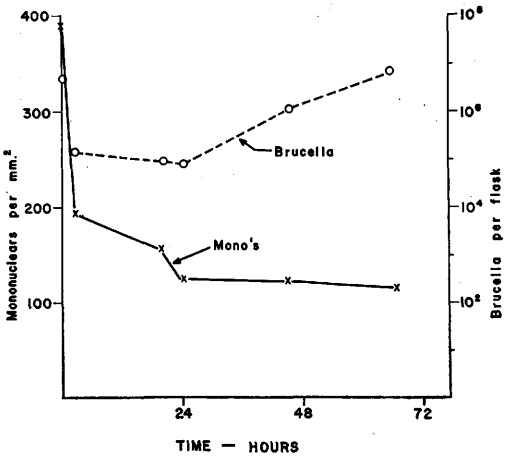

FIG. 2. Shows that there was a definite intracellular multiplication of brucella as evidenced by increase in No. of organisms in cultures while No. of phagocytes remained approximately the same. Note that "0" time shown here is time of beginning of experiment. Medium replaced at 2 hr.

rich in mononuclear phagocytes, were obtained by injection of a dilute solution of glycogen(2,3). Cells were harvested 5 days after injection of glycogen, using heparinized Hanks' solution, centrifuged, washed once with plain Hanks' solution and resuspended in medium composed of 70% Hanks' and 30% normal guinea pig serum to give a concentration of approximately 1 million cells per ml. Living, smooth *Br. abortus* organisms (stain - Lo), from 48-hour-old cultures on tryptose agar, were added to medium in proportion of 50 bacteria per cell. One and a half ml aliquots of mixture were transferred to Porter flasks containing flying coverslips and incubated 2 hours. This time was sufficient for leucocytes to settle and ingest the

TABLE I. Distribution of Intracellular *Br. abortus* among 50 Mononuclear Phagocytes in Stained Coverslip Preparations, at Different Intervals after Replacing Supernatant with Streptomycin Containing Medium.

Hr after replacing medium	Mode (No. of *Brucella abortus* most common in each of 50 phagocytes)
0	1–2
2	1–2
20	7–8
26	11–15
47	25*
68	25*

* This category is arbitrary. Many cells contained brucellae far in excess of 25.

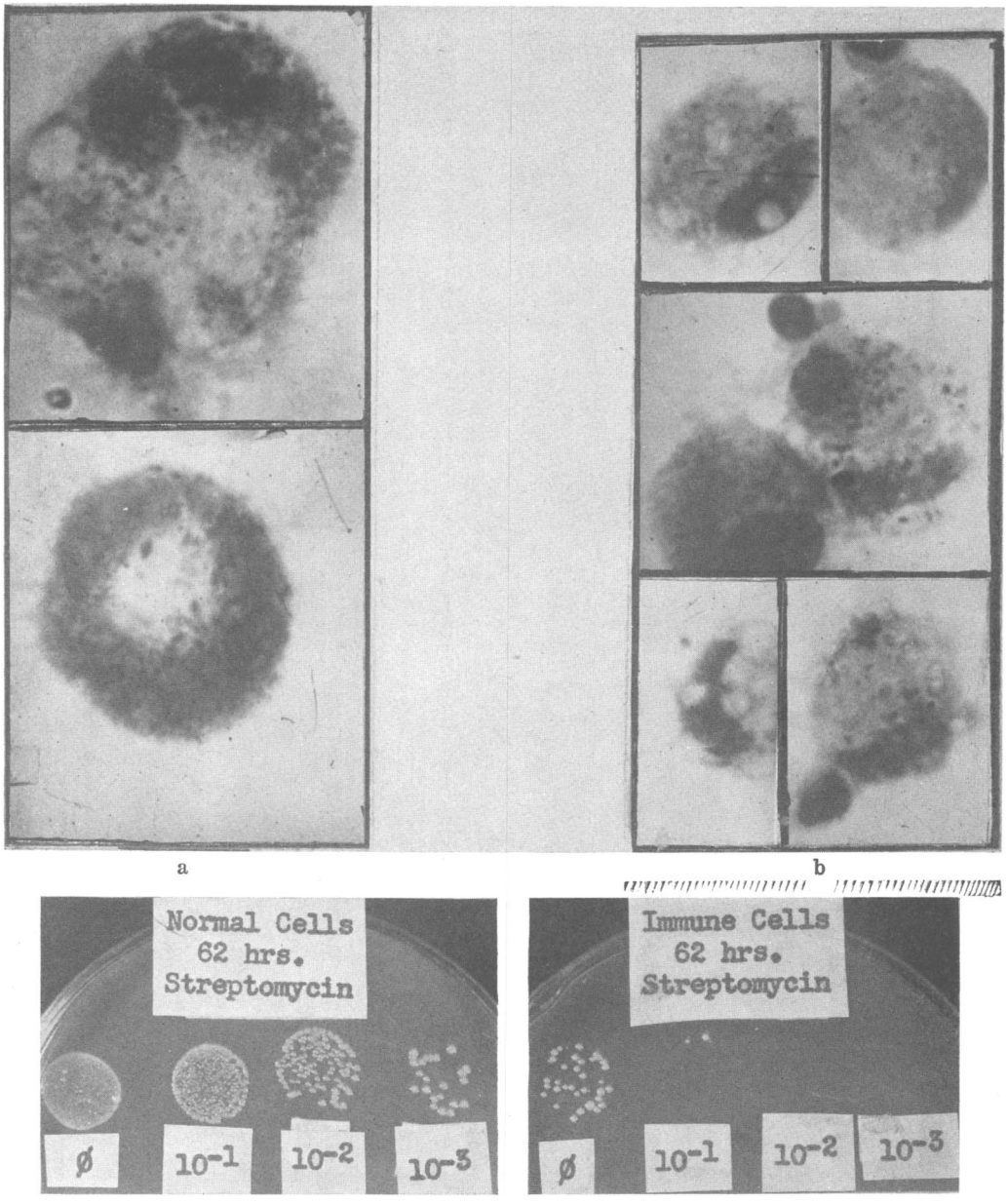

Plate count: 5.75×10^6/flask

Plate count: 1.0×10^4/flask

FIG. 3. Showing difference in No. of intracellular brucellae in normal and immune mononuclear phagocytes from 62-hr-old cultures in medium containing streptomycin. (a) *Normal phagocytes* with large compact masses of brucellae and central clear space with few or no organisms. Notice correspondingly high counts in cultures from same flask. b. *Immune cells* with dark cytoplasm (intense diffuse pink in color prints) and few intact organisms. Notice vacuolation of cells and correspondingly low colony count.

bacteria. After this preliminary incubation supernatant was replaced with fresh cell-free medium containing 10 μg of streptomycin per ml, which was enough to kill all extracellular brucellae without producing noticeable effects on the leucocytes or intracellular organisms. The medium was pipetted off at various times and coverslips removed,

stained by Machiavello's technic or Jacobson's modification of May-Grunwald-Giemsa (1-a), and number of intracellular brucellae in each of 50 cells counted. Leucocytes remaining in flask were scraped off with a rubber policeman and washed twice with Hanks' solution. Cells were resuspended in 5 ml Hanks', transferred to special cups, shaken in Mickle tissue disintegrator at optimal amplitude for 2 minutes, and bacterial counts made by modification(4) of surface plate drop method of Miles and Misra(5). Relative counts of mononuclears in flask were made using flying coverslips. The number of cells per mm^2 were counted using an ocular micrometer. These counts gave only relative numbers but these were sufficient to determine the trend. We were interested in whether or not the number of tissue cells were fairly constant during the period of multiplication of the bacteria. This count acts to control such factors as reingestion of bacteria and differential disruption of sensitive cells. The initial drop in numbers of cells (Fig. 2) was seen whether or not the infectious agent was added to the cultures.

Results with normal monocytes. The results of a typical experiment using mononuclear phagocytes from normal guinea pigs are summarized in Figs. 1 and 2 and Table I.

The data show that viable brucellae increased approximately one hundred fold while phagocytic cell count remained almost constant. During the same time the stainable intracellular brucellae also increased markedly. Even with such great increase of intracellular bacteria tissue cells tolerated the organisms fairly well.

Results with immune mononuclear phagocytes. A comparison of findings with normal and immune mononuclear phagocytes is shown in Fig. 3 (a, b) and Tables II and III. We must emphasize the fact that mononuclear cells, obtained from guinea pigs which had been previously inoculated with living brucellae, always remained sterile after one week incubation in Hanks-serum medium and stained preparations from these cultures did not contain intracellular organisms. Intracellular brucellae always derived from or-

TABLE II. Comparison between Intracellular Growth of *Br. abortus* in Normal Guinea Pig Phagocytes (NC) and in Phagocytes Obtained from Guinea Pigs Which Had Been Inoculated with *Br. abortus* 2 Months Previously (IC).

Hr after change of medium	Kind of cells	Colony count/flask	Distribution of brucella among 50 monocytes in stained coverslips preparations									Remarks
			0	1–2	3–4	5–6	7–8	9–10	11–15	16–25	>25	
0	NC	4.75×10^5	22	11	10	5	1	1	0	0	0	About 50% of cells contained small numbers of brucellae. No apparent disintegration of intracellular organisms.
	IC	5.5 "	30	8	7	4	1	0	0	0	0	Same as for normal cells.
20	NC	1.5×10^4	0	3	4	9	5	4	12	5	8	
	IC	7.5×10^3	2	2	4	10	4	6	7	7	8	
40	NC	5.0×10^4	0	3	1	6	6	3	8	5	19	6 of 50 cells loaded with brucella. Few cells showed pale pink color of cytoplasm.
	IC	7.5×10^2	0	5	5	6	7	3	12	6	6	Many cells showed moderate pink coloration of cytoplasm.
62	NC	5.75×10^6	0	2	2	6	5	5	10	4	16	8 of 50 cells counted loaded with brucella. Some slight pink coloration of cytoplasm.
	IC	1.0×10^1	0	5	6	7	3	3	8	4	14	One of 50 cells loaded with brucella. Majority of cells showed marked diffuse pink coloration of cytoplasm.

This experiment was repeated 4 times with similar results.

TABLE III. Comparison between Intracellular Growth of *Br. abortus* in Normal Phagocytes (NC) from Normal Guinea Pigs 173 and 174 and in Immune Phagocytes (IC) from Guinea Pig (No. 125) Which Had Been Inoculated with *Br. abortus* 16 Months Previously.*

Hrs after change to streptomycin containing medium	Cells	No. of colonies of brucellae/flask
28	NC (173)	2.5×10^5
	IC (125)	5.0×10^4
	NC (174)	3.0×10^5
48	" (173)	8.7×10^5
	IC (125)	3.0×10^4
	NC (174)	9.0×10^5
62	" (173)	4.5×10^6
	IC (125)	7.5×10^4
	NC (174)	1.2×10^6

* Preliminary experiments with mononuclear phagocytes from 3 guinea pigs 10 days after inoculation with living brucellae, and from 2 animals 18 mo after infection, gave in each case results comparable to those obtained with cells from normal controls.

ganisms added to the medium. These experiments show that brucellae within immune mononuclear phagocytes do not reach the numbers they do within normal cells. There is good evidence that some destruction of bacilli takes place within the cells and that this results in a pink staining of the cytoplasm presumably due to products of bacterial disintegration.

In preparations stained by Machiavello's method and in color prints, differences between normal and immune phagocytes are prominent. Intact intracellular brucellae and their products of disintegration stain red, while the host cell stains blue.

Discussion. *Brucella abortus* has long been thought to be capable of intracellular parasitism. This study demonstrates that brucellae can survive phagocytosis and multiply within mononuclear phagocytes of normal guinea pigs maintained *in vitro*. The mononuclear host cells do not show any prominent evidence of injury during a 3-day period of intracellular growth of *Br. abortus*. However, disruption of the cells takes places when the bacilli reach such numbers as to seem capable of mechanically bursting the phagocytes. The observations made during this study do not suggest that any potent toxic material is being released by the bacteria.

Cells obtained from infected (immune) guinea pigs seem capable of either destroying intracellular brucellae after intracellular multiplication has taken place or of suppressing intracellular growth. Most cells were infected with few bacilli during the preliminary incubation period and the outcome must depend on the degree of resistance of the host cell, the virulence of the parasite and the degree of sensitivity of the phagocyte to brucellae and their products. Differences in virulence could be an important factor, as pointed out by Braun,‡ because we are most probably using heterogenous cultures composed of individual cells of different virulence. This may explain why, even after prolonged incubation, there are always some cells which seem to have very few intracellular organisms.

This study indicates that guinea pig mononuclear phagocytic cells and *Brucella abortus* constitute a good model system for the study of host-parasite relationships at the cellular level. In general we have reached similar conclusions as Lurie(6) and Suter(2,3) who worked with *Mycobacterium tuberculosis*. The brucellae, however, offer obvious technical advantages over *Mycobacterium tuberculosis*, in studies of this nature. The brucella-phagocyte system is more amenable to quantitative manipulation.

Summary. The results of typical experiments in host-parasite relationships at cellular level, using *Brucella abortus*-mononuclear phagocyte systems, have been presented. The intracellular multiplication of *Br. abortus* in normal guinea pig mononuclear phagocytes was observed. In *Brucella abortus*-immune mononuclear phagocyte systems the multiplication of the organisms was significantly restricted.

‡ Brown, W., Personal communication.

1. Leighton, J., and Hanks, J. H., *An Introduction to Cell and Tissue Culture,* Chap. 12. By the Staff of Tissue Culture Course, 1949-53, Burgess Publishing Co., 1955.

1-a. *ibid.,* p120.

2. Suter, E., *J. Exp. Med.,* 1952, v96, 137.

3. ———, *ibid.,* 1953, v97, 235.

4. Pomales-Lebrón, A., and Fernández, C., *J. Bact.,*

1952, v64, 837.
5. Miles, A. A., and Misra, S., *J. Hyg. (camb.)*, 1938, v38, 732.
6. Lurie, M. B., *J. Exp. Med.*, 1942, v75, 247.

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DNA Synthesis and Incorporation of P^{32} in Irradiated Ehrlich Ascites Cells.* (22861)

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The experiments to be reported are part of a series of studies on the disturbances of desoxyribose nucleic acid (DNA) metabolism after irradiation. The interpretation of biochemical changes observed in irradiated mammalian tissues is complicated by the fact that they are generally composed of mixed cell populations(1). To circumvent this difficulty, many investigators have recently used ascites tumors since they can be obtained as almost pure cell suspensions(2,3,4).

It has been suggested(5,6,7) that while incorporation of precursors into DNA normally occurs only at the time of DNA synthesis, incorporation after irradiation might be due to turnover of existing DNA not accompanied by net synthesis. This seemed an important point which warranted further investigation.

Materials and methods. Ehrlich ascites tumor used was kindly donated by Dr. E. N. Sassenrath and had been carried in C_3H mice for several years. For each experiment 20 to 30 C_3H mice were inoculated intraperitoneally with ascitic fluid containing approximately 2 x 10^7 cells. All measurements were carried out between the fourth and sixth day after transplantation. Irradiated and control groups were always run simultaneously, and a minimum of 5 but frequently 10 mice were used for each point. The total body irradiation (800 r or 1400 r) was carried out as previously described(1). Cell number per mouse for the growth curves was calculated from the total volume of ascitic fluid and cell concentration in the ascitic fluid. To determine the volume of ascitic fluid 2 μc of radioiodinated (I^{131}) serum albumin (RISA, Abbott Laboratories) were injected intraperitoneally after the mouse had been sacrificed. After a 3 minute mixing period, ascitic fluid was withdrawn and counts in a 20 μl aliquot were compared to the injected dose. Cell concentration was determined by counting duplicate dilutions in a hemocytometer. DNA content and total nucleic acid content were determined in duplicate aliquots of ascitic fluid from each mouse. After the cells were centrifuged, their nucleic acids were extracted by the hot perchloric acid method of Ogur and Rosen(8). Total nucleic acid content of the extract was determined by ultraviolet absorption and the DNA content by Ceriotti's indole method(9). For calculation of mean nucleic acid content per cell the concentration of tumor cells was determined by "large cell counts"(10) in a hemocytometer. Since the proportion of non-tumor cells was consistently low (around 5% as determined by enumeration of small cells in the hemocytometer or differential counts on stained smears), no correction was made for their presence. Mean cell volume was calculated from the cell concentration and the "hematocrit" as measured in micro-hematocrit capillary tubes. Separate groups of mice were used for the incorporation studies. They were injected intraperitoneally with 20 μc $Na_2HP^{32}O_4$ at varying times after irradiation together with unirradiated controls and sacrificed 2 hours after injection. Ascitic fluid from 2 mice was pooled, and the cells sepa-

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