A CELLULAR BASIS OF IMMUNITY IN EXPERIMENTAL BRUCELLA INFECTION*

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Plates 21 to 24

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Brucellae are known to be facultative intracellular parasites (1, 2). Braude (2) showed that within 24 hours after infection of guinea pigs with *Brucella abortus*, nearly all brucellae detectable in the peripheral blood were within neutrophiles. These infected phagocytes (and extracellular bacteria) were removed from the circulation by the spleen, liver, and other organs. Focal aggregations of parasitized polymorphonuclear and mononuclear phagocytes in these organs led to development of granulomas. Phagocytized brucellae were not destroyed but rather appeared to multiply intracellularly until many phagocytes were completely engorged. With onset of the mature granuloma, marked by the appearance of epithelioid cells, microscopically evident brucellae gradually disappeared from the tissues.

Intracellular multiplication of *Brucella in vitro* has recently been demonstrated (3, 4). Similar methods (5–7), and methods involving passive transfer of cells or humoral factors (8, 9), have been applied to the study of acquired immunity in tuberculosis, but with equivocal results. Acquired resistance in brucellosis is much more amenable to investigation because high titer agglutinating antibody can be produced, and because reproducible viability counts from infected cells and tissues are possible.

The studies reported here were initiated to determine the reasons for the restricted multiplication of brucellae within phagocytes of animals previously infected with *Brucella*. While this work was in progress, others (4, 10) reported that monocytes from vaccinated guinea pigs and rabbits restricted intracellular growth of brucellae in a cell culture system, whereas marked multiplication occurred within normal monocytes under the same conditions. Our findings confirm and extend their results.

Materials and Methods

Animals.—Three species were used: male guinea pigs (350 to 500 gm.), female rats of the Sprague-Dawley strain (250 gm.), and young adult (about 20 gm.) mice of both sexes (inbred progeny of LAF_1 strain of mice).

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Bacterial Cultures.—Brucellae were grown and maintained on *Brucella* agar (Albimi). The smooth strains used were *Brucella abortus* strain a77, strain a5 (CO_2-dependent), and strain 19 (Lederle No. 1952a); *Brucella suis* strain s101; and *Brucella melitensis* strain m62. Non-smooth variants of brucellae were obtained by prolonged growth of a smooth inoculum in *Brucella* broth (Albimi) containing 300 μg. per ml. DL-α-alanine. The acriflavine test (11) was used in conjunction with antiserum and with colonial observation by oblique light (12) for selection of mucoid and rough variants.

Brucellae used for infection of monocytes or animals were harvested from the surface of *Brucella* agar after 2 days' growth, and suspended to the desired concentrations in sterile distilled water by adjustment of density to match barium sulfate standards.

Vaccination of Animals.—Hereafter, animals infected as described below will be referred to as immunized. They were used between 4 and 10 weeks postinfection. Guinea pigs were injected intraperitoneally with approximately 10^8 living, smooth, virulent *B. abortus* (strain a77). Mice received approximately 10^8 smooth *B. melitensis* subcutaneously.

When specified below, heat-killed (60°C. for 1 hour) brucellae were employed for vaccination, skin testing, and desensitization.

Skin Testing and Desensitization.—Animals were skin tested for delayed hypersensitivity by intracutaneous injection of 0.1 ml. brucellergen (Sharp & Dohme) or 0.1 ml. of a suspension of heat-killed *B. suis* containing 10^8 organisms per ml. Within several weeks intracutaneous injections failed to elicit a delayed reaction, and the animals were considered to be desensitized.

Monocyte Preparations.—Monocyte preparations were made according to a modification of the procedure of Barski *et al.* (13), using 16 × 125 mm. screw-cap test tubes coated on the inside with formvar (14). Glycogen was used as a chemotactic agent for the induction of peritoneal exudates. Oyster glycogen (Eastman), 0.002 per cent in physiological saline solution, was injected intraperitoneally once daily for 4 days. Guinea pigs received 15 ml. daily, rats 10 ml., and mice about 1.5 ml. Peritoneal exudates were harvested aseptically on the 5th day in Hanks's balanced salt solution (BSS) containing 5 units per ml. heparin. The BSS was injected intraperitoneally (60 ml. into guinea pigs, 50 ml. into rats, and about 8 to 10 ml. into mice). Their abdomens were lightly massaged for several minutes to suspend exudate cells in the BSS. Mice and rats were sacrificed with chloroform and guinea pigs were anesthetized with ether. The BSS exudates were withdrawn through a 16 gauge perforated needle and placed into a silicone-coated, sterile, screw-cap test tube. The cells were sedimented by centrifugation and washed twice in BSS containing 20 per cent normal rabbit serum. The first supernatant BSS was saved and used later in the medium ("conditioned BSS" of Barski *et al.* (13)). If it came from vaccinated animals, however, it was discarded since antibody was often present.

The washed guinea pig and mouse cells were suspended in complete medium to a concentration of about 1.5 million cells per ml. Because of their smaller size, rat exudate cells were adjusted to a concentration of about 3 million cells per ml. These 5 day exudates from all three species consisted mainly of mononuclear phagocytes. Exudates grossly contaminated with red blood cells were discarded. For convenience, the mononuclear phagocytes will be described here as monocytes although their morphology in culture was often more suggestive of histiocytes (or macrophages). Mouse monocytes were pooled before culturing.

The monocyte culture medium was composed of 55 per cent conditioned BSS, 40 per cent rabbit serum, and 5 per cent chick embryo extract. Pooled normal rabbit serum inactivated at 56°C. for 30 minutes was used unless otherwise indicated. Inactivated rabbit serum was found to be equal or superior to homologous sera if conditioned BSS and 5 per cent chick embryo extract were also used in the medium.

One ml. of monocyte suspension in the complete medium was placed into each culture tube. The monocytes were immediately infected by addition to the cell suspension of approximately 2×10^7 brucellae. Tubes containing monocytes and bacteria in suspension were then incubated

for 5 hours at 37°C. in an almost horizontal, stationary position. During this time the monocytes settled to the lower walls of the tubes and became firmly adherent to the formvar film.

Following the 5 hour infection period the medium in each tube was discarded and the tubes were rinsed twice with BSS containing 20 per cent normal rabbit serum to remove most nonadherent leucocytes, erythrocytes, and extracellular bacteria. One and one-half ml. of fresh complete medium containing 10 μg. per ml. streptomycin sulfate (to prevent extracellular growth of bacteria) was then deposited in each tube. At this time the lower wall of each tube contained a monolayer of monocytes many of which could be observed to contain one or more intracellular bacteria when examined microscopically in stained coverslip preparations. The tubes were incubated in a nearly horizontal, stationary position at 37°C. One-half the medium was removed and replaced with fresh medium whenever the pH dropped below 7.2 in any tube.

The intracellular growth of brucellae was followed by microscopic observation and by quantitative cultural techniques. For the former, formvar-coated coverslips were included in some of the tubes, withdrawn at intervals, and stained by Perrin's modification of the Goodpasture stain (15). For the latter, plate counts of viable intracellular brucellae per cell culture tube were made at various times after the addition of streptomycin-containing medium. This was effected by first removing and discarding the medium in each tube, and replacing this with 1.0 ml. of sterile distilled water. The adhering monocytes were then scraped from the walls of the tube and suspended in the water. This suspension was thoroughly ground in a sterile Ten-Broeck grinder to disrupt the cells and disperse intracellular brucellae. Finally, one-tenth ml. aliquots of serial tenfold dilutions of this suspension were plated in duplicate on the surface of *Brucella* agar.

To determine approximate percentage loss of monocytes during the 3 day infection period, coverslips were removed from culture tubes at 5 and 72 hours after addition of streptomycin, and were examined under the high power objective of the microscope (× 430). The number of cells was counted for each of three representative fields, and the average recorded. Those occasional cultures showing gross, early sloughing of cells from the walls of the tube were discarded.

EXPERIMENTAL OBSERVATIONS

Growth of Smooth Brucellae in Normal Monocytes.—Most of the monocytes from normal animals survived *in vitro* for at least 3 days. Those from guinea pigs and rats retained their basic morphology for 3 days *in vitro*, whereas those from mice gave rise to many spindle-shaped cells (Fig. 1) after the 1st day of incubation. These did not appear to result from proliferation of contaminating fibroblasts.

Smooth brucellae of all three species grew within normal mouse and guinea pig monocytes as determined microscopically (Figs. 2 and 3) and by viability counts of bacteria from disrupted monocytes (Table I). The count of viable *Brucella* per culture tube reached a maximum by the 3rd day and then usually declined rapidly as the heavily infected monocytes degenerated and allowed contact between the brucellae and the streptomycin in the medium. Infected monocytes survived as well as uninfected cells for the first 3 days but thereafter they rounded up and sloughed off the walls of the tubes in large numbers. It is apparent from Figs. 2 and 3 that normal monocytes exhibited little or no cellular reaction to the infecting *Brucella*. It is striking that some monocytes

were literally packed with brucellae while adjacent monocytes were either lightly infected or uninfected.

Growth of Smooth Brucellae in Monocytes from Immunized Animals.—Monocytes derived from animals previously infected (immunized) with smooth brucellae were examined for ability to support intracellular growth of smooth *B. suis*. Chart 1 compares the growth of *B. suis* in normal and "immune" monocytes in representative experiments. The brucellae multiplied well within monocytes from all species of normal animals but only poorly within cells from previously infected animals. Guinea pig 5 was the only immunized animal

TABLE I

Growth of Smooth Brucellae within the Monocytes of Guinea Pigs and Mice in Tissue Culture

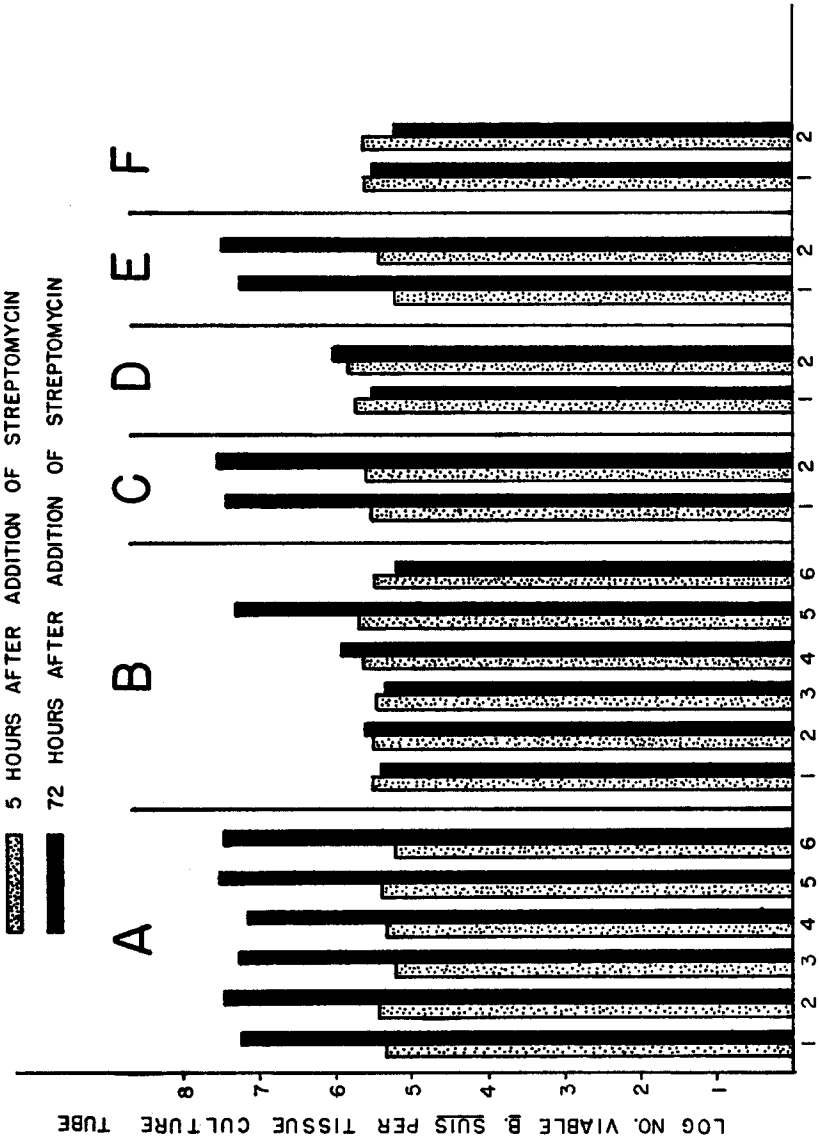
Infecting organism	Viable *Brucella* per culture tube at various hours after addition of streptomycin-containing medium				
	5	24	48	72	96
Normal guinea pig monocytes					
B. suis	4×10^5	1×10^6	8×10^6	6×10^7	2×10^7
B. abortus	2×10^5	5×10^5	4×10^6	4×10^7	9×10^6
B. melitensis	3×10^5	9×10^5	9×10^6	2×10^7	8×10^6
Normal mouse monocytes					
B. suis	7×10^5	2×10^6	1×10^7	6×10^7	9×10^6
B. abortus	6×10^5	9×10^5	7×10^6	2×10^7	1×10^7
B. melitensis	5×10^5	1×10^6	8×10^6	4×10^7	8×10^6

tested in this laboratory whose monocytes failed to inhibit intracellular growth of *Brucella*.

Figs. 4 and 5 show the reaction of "immune" guinea pig monocytes to infection with smooth *B. suis*. The cells exhibit a foamy cytoplasm with relative isolation of the brucellae one from another, rather than bacterial colony formation, which occurred within most normal cells similarly infected. The faintly staining granules in the cytoplasm may be brucellae undergoing destruction. A similar picture was seen in infected monocytes from immunized mice (Fig. 6) and rats.

Not all the monocytes in cultures from immunized animals restricted multiplication of intracellular *Brucella*. Although most "immune" monocytes were uninfected or lightly infected after 3 days, a few were moderately infected, and occasionally heavily infected cells could be found.

The Survival in Vitro of Infected Monocytes from Normal and Immunized Animals.—It was important to determine whether the restricted growth of *Brucella* within "immune" monocytes might be a consequence of hastened



A, monocytes derived from normal guinea pigs.
B, monocytes derived from immunized guinea pigs.
C, monocytes derived from normal mice.
D, monocytes derived from immunized mice.
E, monocytes derived from normal rats.
F, monocytes derived from immunized rats.

CHART 1. Growth of *B. suis* within normal and "immune" monocytes cultured and infected *in vitro*. "Immunized" animals were infected with living virulent brucellae between 4 to 10 weeks before removal of their monocytes into cell cultures. Each numbered pair of bar graphs represents data derived from monocytes of individual guinea pigs and rats, or from monocyte pools from mice.

death of these phagocytes as compared to normal monocytes. Table II shows the average per cent loss of cells between 5 and 72 hours' incubation in three typical experiments. It can be seen that both normal and "immune" monocytes survived about equally well during the 3 day infection period *in vitro*. The viability of those monocytes remaining adherent to the tubes after 3 days was confirmed by demonstrating their ability to phagocytize heat-killed Gram-positive bacteria added to the cultures at this time.

Effect of Brucella Antiserum.—Although the "immune" monocytes used above were washed several times before infection, it was considered possible that their inhibitory activity against brucellae might be due to antibody remaining on or in the cells. Therefore the effect of antiserum on the growth of

TABLE II

Per Cent Loss of Normal and "Immune" Monocytes during 3 Days' Cultivation in Vitro after Infection with B. suis

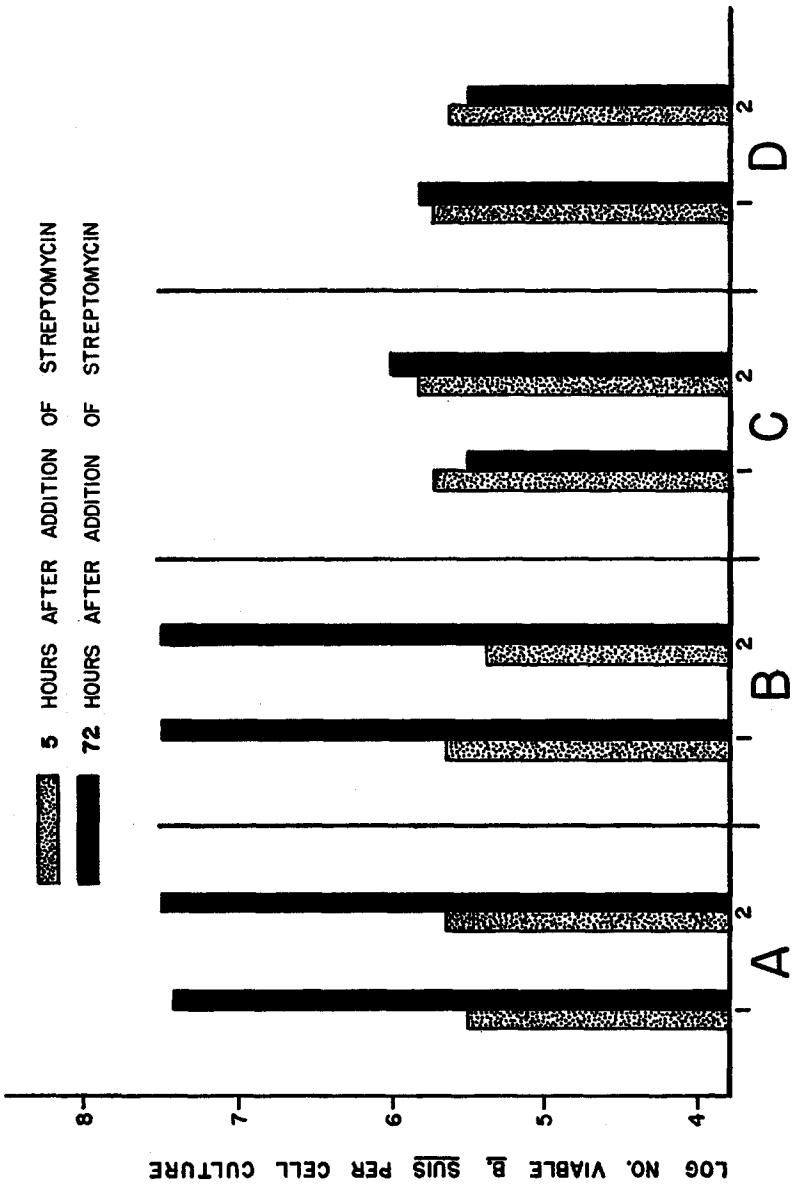
Monocytes derived from	Average No. monocytes per microscope field at		Average per cent loss in 3 days
	5 hrs.	72 hrs.	
Normal guinea pigs	189	132	30
"Immune" guinea pigs	203	129	36
Normal mice	192	129	33
"Immune" mice	177	104	41
Normal rats	513	323	37
"Immune" rats	481	285	41

brucellae within normal and "immune" monocytes was determined. A mixture of 80 per cent rabbit *Brucella* antiserum[1] (agglutinating titer greater than 1/1280) and 20 per cent mouse *Brucella* antiserum[1] (titer 1/120) was substituted for the normal serum in the cell culture medium. The presence of these antisera (not heat-inactivated) had no detectable effect on the growth or survival of brucellae within either normal or "immune" monocytes (Chart 2). Similar results were obtained with guinea pig monocytes using fresh homologous antiserum.

Role of Delayed Hypersensitivity.— The acquired cellular resistance demonstrated above was exhibited by mouse monocytes although mice do not develop classical delayed (tuberculin type) skin reactions even in the presence of progressive tuberculosis (16). Similarly, we could not elicit a positive skin test in *Brucella*-infected mice, using either brucellergen or dead *Brucella* cell antigens.

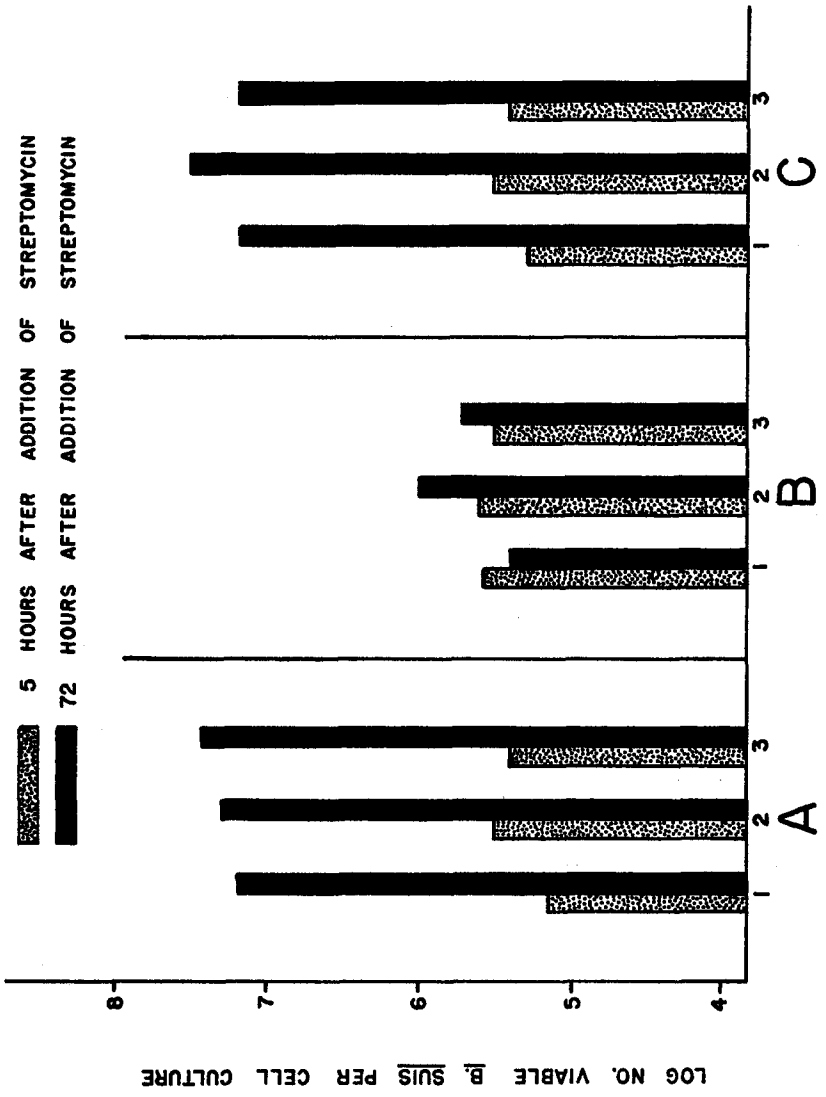
Brucella-infected guinea pigs gave positive delayed skin tests with both

[1] Pooled serum from animals heavily infected several months previously with smooth brucellae.



A, monocytes from normal mice. Normal serum added.
B, monocytes from normal mice. Antiserum added.
C, monocytes from immunized mice. Normal serum added.
D, monocytes from immunized mice. Antiserum added.

CHART 2. Lack of effect of *Brucella* antiserum, in the cell culture medium, on the growth of *B. suis* within either normal or "immune" mouse monocytes cultured *in vitro*.



A, monocytes from normal guinea pigs.
B, monocytes from immunized, desensitized guinea pigs.
C, monocytes from guinea pigs vaccinated with heat-killed *B. suis*.

CHART 3. Growth of *B. suis* within monocytes derived from normal guinea pigs, from immunized, desensitized guinea pigs, and from guinea pigs vaccinated 1 to 2 months previously with heat-killed brucellae. The heat-killed brucellae were injected intraperitoneally with a single dose containing approximately 5×10^9 smooth *B. suis*. Animals were desensitized by bi-weekly intracutaneous injections of heat-killed *B. suis*.

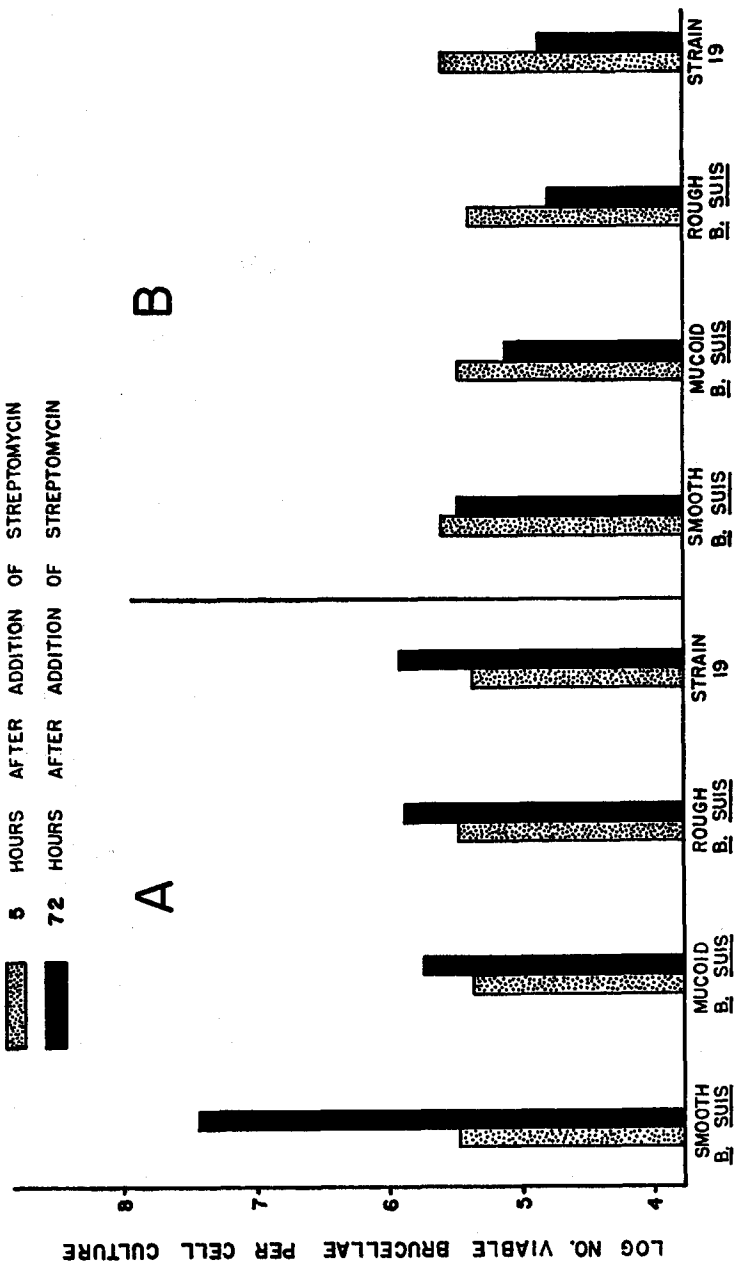
brucellergen and dead cell antigens. Guinea pigs vaccinated with heat-killed smooth brucellae did not develop delayed hypersensitivity although most produced high titers of agglutinating antibody. Two techniques were therefore available for demonstrating a relationship, if such exists, between acquired cellular resistance and delayed hypersensitivity; namely, (1) desensitization of *Brucella*-infected guinea pigs, and (2) vaccination of normal guinea pigs with dead *Brucella*. Monocytes were removed from animals treated by the above methods, and were infected *in vitro*. The results shown in Chart 3 indicate that desensitization did not detectably alter the ability of monocytes of infected animals to retard intracellular growth of *Brucella*. It can also be seen in Chart 3 that monocytes derived from guinea pigs vaccinated with dead *Brucella* failed to inhibit intracellular multiplication of brucellae. All three of these animals had high serum titers of agglutinating antibody.

Growth of Non-Smooth Brucella Variants and Strain 19 within Normal and "Immune" Monocytes.—Smooth and non-smooth brucellae have not been shown to be antigenically related by conventional serological methods (17), although there is a definite cross-reaction (delayed hypersensitive skin reaction) to mucoid variant antigens in guinea pigs infected with the smooth type (18). The specificity of growth inhibition by "immune" monocytes was investigated by comparing intracellular growth of smooth and non-smooth variants of *B. suis*. The non-smooth variants did not cross-react with the smooth parent type in agglutination tests.

Chart 4 shows that rough and mucoid *B. suis* variants and strain 19 multiplied slowly in normal mouse monocytes, but decreased in number within "immune" cells during the 3 days. These results are typical of those obtained with both mouse and guinea pig monocytes infected with non-smooth strains. Some intermediate and some frankly non-smooth *Brucella* have been encountered which multiply within normal monocytes at the same rate as do the smooth parent strains. Nevertheless, in every case the "cellular immunity" induced by infection with smooth brucellae has extended at least partially to the serologically unrelated variants.

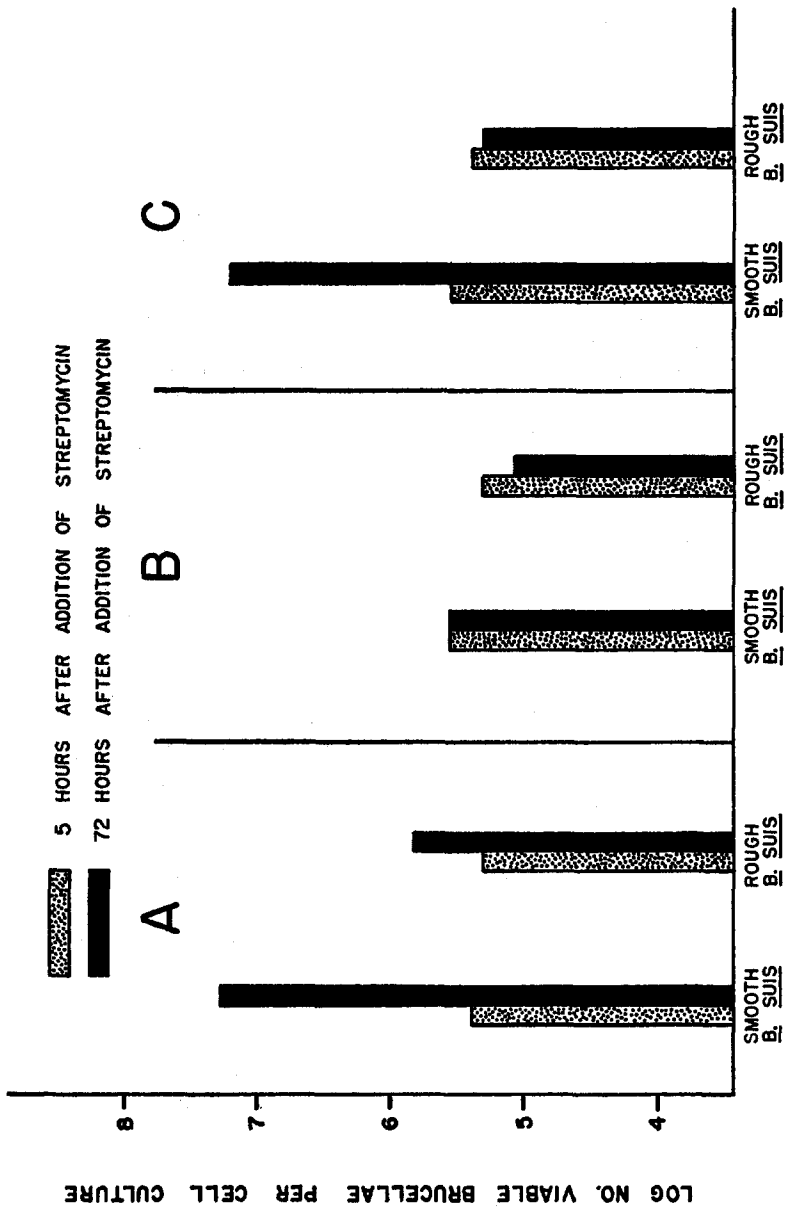
Effect of Vaccination with Living, Rough Brucellae.—The results obtained above (Chart 4) suggested the possibility that "cellular immunity" against smooth *Brucella* might be induced by vaccination with a non-smooth mutant. Chart 5 shows that previous infection of guinea pigs with rough brucellae did not render their monocytes refractory to *in vitro* infection by the smooth parent strain. These animals developed delayed hypersensitivity to brucellergen and to cell antigens of both smooth and rough strains. It can also be seen in Chart 5 that monocytes derived from guinea pigs infected with rough variants did prevent intracellular multiplication of the homologous rough strain.

In vivo Studies of "Cellular Immunity" to Brucellosis in Mice.—It was expected that since "cellular immunity" appeared to be very marked in monocytes



A, monocytes derived from normal mice.
B, monocytes derived from immunized mice.

CHART 4. Results of a typical experiment comparing the growth of smooth and non-smooth brucellae and strain 19 within normal and "immune" mouse monocytes cultured *in vitro*. Note the relatively poor growth of non-smooth brucellae and strain 19 in normal monocytes (A), and the restricted growth of all brucellae within monocytes from mice immunized (infected) with smooth *B. suis* (B).



A, monocytes from normal guinea pigs.
B, monocytes from immunized guinea pigs.
C, monocytes from guinea pigs infected with live, rough *B. suis*.

CHART 5. Growth of smooth and rough *B. suis* within monocytes from normal guinea pigs (A), from guinea pigs infected 1 to 2 months previously with about 10^8 smooth *B. abortus* (B), and from guinea pigs infected 1 to 2 months previously with 5×10^8 rough *B. suis* (C). Results averaged from three separate experiments. Note that monocytes derived from the animals vaccinated with rough brucellae failed to inhibit intracellular growth of smooth brucellae.

in vitro, it should also be demonstrable *in vivo*. We contrived to study *Brucella* infection of mice under conditions which would limit the survival of bacteria to those in an intracellular and/or intragranulomatous site. Since streptomycin destroyed extracellular but not intracellular brucellae *in vitro* it was assumed that it would exert a similar action *in vivo*.

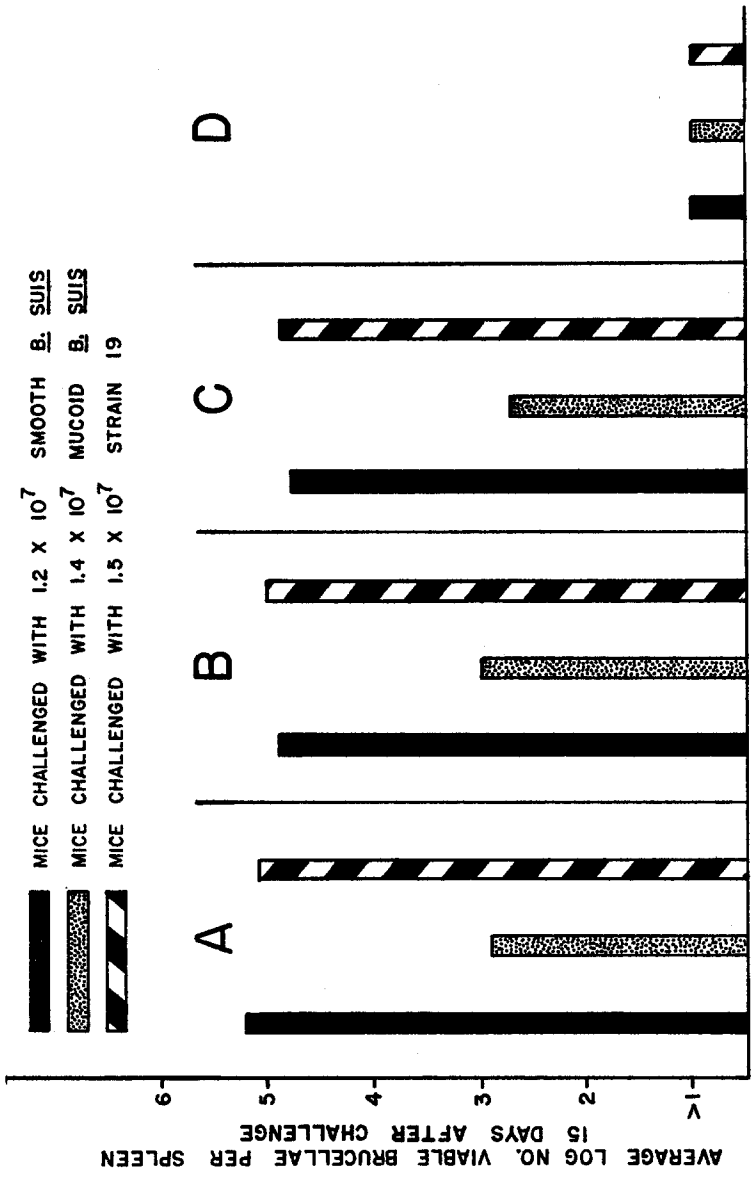
Normal control mice and mice previously vaccinated in various ways were challenged with smooth *B. suis*, then treated daily with a massive dose of streptomycin to prevent extracellular survival or growth of brucellae. The mice were sacrificed 2 weeks after challenge and quantitative determinations were made of viable brucellae remaining within the spleen.

Four groups of mice were challenged: (A) untreated normal controls; (B) mice injected intraperitoneally 1 day previously with 1 ml. of pooled rabbit anti-*Brucella* serum; (C) mice vaccinated 5 weeks previously with approximately 5×10^9 heat-killed *B. abortus* strain a5; and (D) mice immunized (infected) 5 weeks previously with approximately 1×10^8 living, smooth, virulent *B. abortus* strain a5 (CO_2–dependent).

Mice from each of the above groups were challenged by intraperitoneal injection of about 10^7 smooth or mucoid *B. suis*, or strain 19, in saline. Twelve hours later and once daily for 14 days thereafter, each mouse received 1 mg. streptomycin sulfate solution intramuscularly. All mice were sacrificed on the 15th day and their spleens were aseptically removed to sterile test tubes containing a layer of sand on the bottom. Each spleen was thoroughly ground in the sand using a sterile glass rod, and the volume of spleen mash in each tube was brought to 2.0 ml. with sterile distilled water. Tenfold serial dilutions of the mince were spread in 0.1 ml. aliquots on the surface of *Brucella* agar plates. The plates were incubated aerobically at 37°C. to allow growth and enumeration of the remaining challenge brucellae without interference from the CO_2–dependent immunizing strain. As a check against possible development of streptomycin resistance, aliquots were plated on *Brucella* agar containing 20 μg per ml. streptomycin.

Chart 6 shows the survival of *Brucella* strains within spleens of the four groups of mice. Streptomycin-resistant variants did not appear. Surprisingly, strain 19 survived about as well in the normal mice as did smooth virulent *B. suis*, although mucoid *B. suis* were far less numerous. The mice previously immunized by infection with *B. abortus* apparently rid their spleens, with the aid of streptomycin, of all three challenge strains. That this acquired resistance was not merely a manifestation of classical antibody is indicated by the results with mice of groups B and C. Very poor protection was afforded by passively transferred antiserum or by vaccination with heat-killed brucellae. *Brucella* agglutinin titers of 1/80 or above were present in the serum of four of five mice vaccinated with heat-killed brucellae.

If it can be assumed that the streptomycin treatment did limit survival to those organisms within cells, then this experiment provides strong evidence for an acquired cellular resistance acting *in vivo*. As was the case *in vitro*, immunity induced by infection with smooth brucellae extended also to serologically dissimilar mucoid variants. It was to be expected from the results of *in vitro* monocyte infection that *Brucella* antiserum would exert little effect *in vivo* under conditions where streptomycin was allowing only intracellular survival, and this proved to be the case.



A, normal mice.
B, mice treated with 1 ml. *Brucella* antiserum 1 day before challenge.
C, mice vaccinated with heat-killed *B. abortus* 1 month before challenge.
D, immunized mice (infected with living *B. abortus* 1 month before challenge).

CHART 6. *In vivo* experiment showing survival of brucellae within the spleens of normal and vaccinated mice, and mice pretreated with rabbit anti-*Brucella* serum. Twelve hours after challenge infection, and once daily thereafter for 14 days, each mouse received 1 mg. streptomycin intramuscularly to prevent extracellular survival of *Brucella*. All mice were sacrificed on the 15th day and their spleens cultured. Each graph averaged from five mice.

When aliquots of the spleen mince from immunized mice were plated and incubated under 10 per cent CO_2 tension to detect the presence of the CO_2–dependent immunizing strain, only three of the fifteen immunized mice yielded viable brucellae, and in these three animals the viability count was less than 400 *Brucella* per spleen.

Similar results were obtained with viability counts of lymph nodes from the same mice.

DISCUSSION

The mechanism underlying acquired resistance in monocytes remains to be elucidated. This immunity cannot readily be ascribed to classical humoral factors for neither actively nor passively acquired agglutinating antibody evoked "cellular resistance." In addition, it appears to be unrelated to delayed hypersensitivity for we were able to induce delayed hypersensitivity without "cellular immunity" (guinea pigs infected with living rough *B. suis*); and cellular immunity without delayed hypersensitivity (all immunized mice, and immunized, desensitized guinea pigs). It is possible, however, that delayed hypersensitivity may be present at the cellular level without manifesting itself in a skin test. That "cellular immunity" is not an artifact arising from accelerated death of hypersensitive (immune) monocytes is attested by our results. Elberg *et al.* (10) showed that immune monocytes were more refractory to the cytotoxic effect of *Brucella melitensis* than were normal monocytes. The degeneration of infected normal monocytes was rapid in their culture system, in which extracellular bacterial growth was not prevented by incorporation of antibiotic. Both homologous and heterologous (BCG) vaccination protected monocytes from the cytotoxic effects of brucellae. However, Braun *et al.* (19) recently reported that *Brucella* had a greater cytotoxic effect on monocytes from immune animals. We could find no marked differences between the destruction of normal and immune phagocytes by brucellae. These discrepancies might be due to influences of the serum used for cell culture (10) as well as to bacterial strain differences and other variations in procedures.

The "cellular immunity" seen here might represent nothing more than an altered "fauna" of the reticuloendothelial system in which resistant phagocytes, originally present in small numbers, increase relative to the susceptible type as a result of the selective influence of infection. Many monocytes in cultures from normal animals have been shown to restrict intracellular multiplication (Figs. 2 and 3). Conversely, a small proportion of monocytes from immunized animals supported moderate to heavy growth of intracellular *Brucella*. However, if "cellular immunity" was due to an increase in the proportion of natively resistant cell types, it was not reflected in obvious morphologic differences between monocytes from normal and immune animals.

The investigation of "cellular immunity" in intact mice, in which strepto-

mycin therapy was used to prevent extracellular survival of brucellae, gave excellent correlation with *in vitro* results. In both cases cellular immunity could be induced only with living smooth brucellae and was not detectably influenced by the presence of antibody. In both cases immunity elicited by infection with smooth brucellae was effective against non-smooth variants. This is further evidence against a role for non-smooth variants as a reservoir of chronic infection (18, 20), despite their selective advantage in sera from *Brucella*-infected animals (21).

If the reticuloendothelial system of chronically infected animals behaves in the intact animal as monocytes from immunized animals do *in vitro*, a small proportion of phagocytes would allow multiplication of intracellular brucellae. Deaths of these heavily infected cells might on occasion release showers of bacteria into the circulation. Such occurrences would be minimized by the fact that most infected cells of the reticuloendothelial system are not isolated, but are in juxtaposition to other phagocytes within a granuloma in which bacteria released from one cell could be dealt with by other phagocytes. It is obvious that a host-parasite relationship of this sort, in which most but not all host phagocytes are resistant to intracellular growth, could lead to chronic infection with the possibility of clinical relapse. Relapse would hinge on other specific and non-specific host factors and environmental influences on the host. Dubos and Schaedler (22, 23) recently reported both enhancement of staphylococcal infection and protection against infection with products of heterologous bacteria, depending upon time of administration. These effects were apparently due to an alteration in response of host cells.

That factors present in the intact animal contribute to acquired intracellular resistance is indicated by the very marked "cellular immunity" observed in the *in vivo* experiment. "Immune" monocytes *in vitro* merely retarded intracellular multiplication of brucellae, but spleen cells of streptomycin-treated, immunized mice rid themselves of challenge brucellae within 14 days.

SUMMARY

Brucella suis, *Brucella abortus*, and *Brucella melitensis* were shown by microscopic and cultural procedures to multiply extensively within normal rat, mouse, and guinea pig monocytes maintained *in vitro* in cell cultures for 3 days. Intracellular growth of brucellae had no observable toxic effects on most monocytes, although many of the cells became completely engorged with brucellae within 3 days. Non-smooth brucellae and strain 19 multiplied slowly within normal monocytes. In contrast, "immune" monocytes, *i.e.* those derived from animals previously infected with smooth brucellae, greatly restricted the intracellular growth of smooth and non-smooth brucellae and strain 19. Growth of smooth *Brucella*, within either normal or "immune" monocytes, was not influenced by addition of *Brucella* antiserum to the culture medium.

Desensitization of immunized guinea pigs did not diminish the refractory state of their monocytes. Cellular resistance did not develop when animals were vaccinated with heat-killed brucellae, though these animals did produce agglutinating antibody. Similarly, vaccination of animals with living, rough *B. suis* failed to induce a refractory state in their monocytes, even though the vaccinated animals developed delayed hypersensitivity to smooth *Brucella* antigen.

In vivo studies of *Brucella* survival in the spleens of normal and vaccinated mice (treated with streptomycin to prevent extracellular survival) gave strong support to the *in vitro* demonstrations of acquired "cellular immunity."

Some implications of these results are discussed.

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BIBLIOGRAPHY

1. Goodpasture, E. W., and Anderson, K., The problem of infection as presented by bacterial invasion of the chorioallantoic membrane of chick embryos, *Am. J. Path.*, 1937, **13**, 149.
2. Braude, A. I., Studies in the pathology and pathogenesis of experimental brucellosis. II. The formation of the hepatic granuloma and its evolution, *J. Infect. Dis.*, 1951, **89**, 87.
3. Holland, J. J., and Pickett, M. J., The intracellular behavior of *Brucella* variants in chick embryo cells in tissue culture, *Proc. Soc. Exp. Biol. and Med.*, 1956, **93**, 476.
4. Pomales-Lebrón, A., and Stinebring, W. R., Intracellular multiplication of *Brucella abortus* in normal and immune mononuclear phagocytes, *Proc. Soc. Exp. Biol. and Med.*, 1957, **94**, 78.
5. Suter, E., Multiplication of tubercle bacilli within mononuclear phagocytes in tissue cultures derived from normal animals and animals vaccinated with BCG, *J. Exp. Med.*, 1953, **97**, 235.
6. Suter, E., Relation between growth inhibitory property of monocytes for tubercle bacilli and hypersensitivity to tuberculin: An *in vitro* study, *in* Ciba Symposium on Experimental Tuberculosis, (G. E. W. Westenholme and M. P. Cameron, editors), Boston, Little, Brown and Co., 1955, 198.
7. Mackaness, G. B., The growth of tubercle bacilli in monocytes from normal and vaccinated rabbits, *Am. Rev. Tuberc.*, 1954, **69**, 495.
8. Raffel, S., The mechanism involved in acquired immunity to tuberculosis, *in* Ciba Symposium on Experimental Tuberculosis, (G. E. W. Westenholme and M. P. Cameron, editors), Boston, Little, Brown and Co., 1955, 261.
9. Lurie, M. B., The fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals, *J. Exp. Med.*, 1942, **75**, 247.
10. Elberg, S. S., Schneider, P., and Fong, J., Cross-immunity between *Brucella melitensis* and *Mycobacterium tuberculosis*: Intracellular behavior of *Brucella melitensis* in monocytes from vaccinated animals, *J. Exp. Med.*, 1957, **106**, 545.

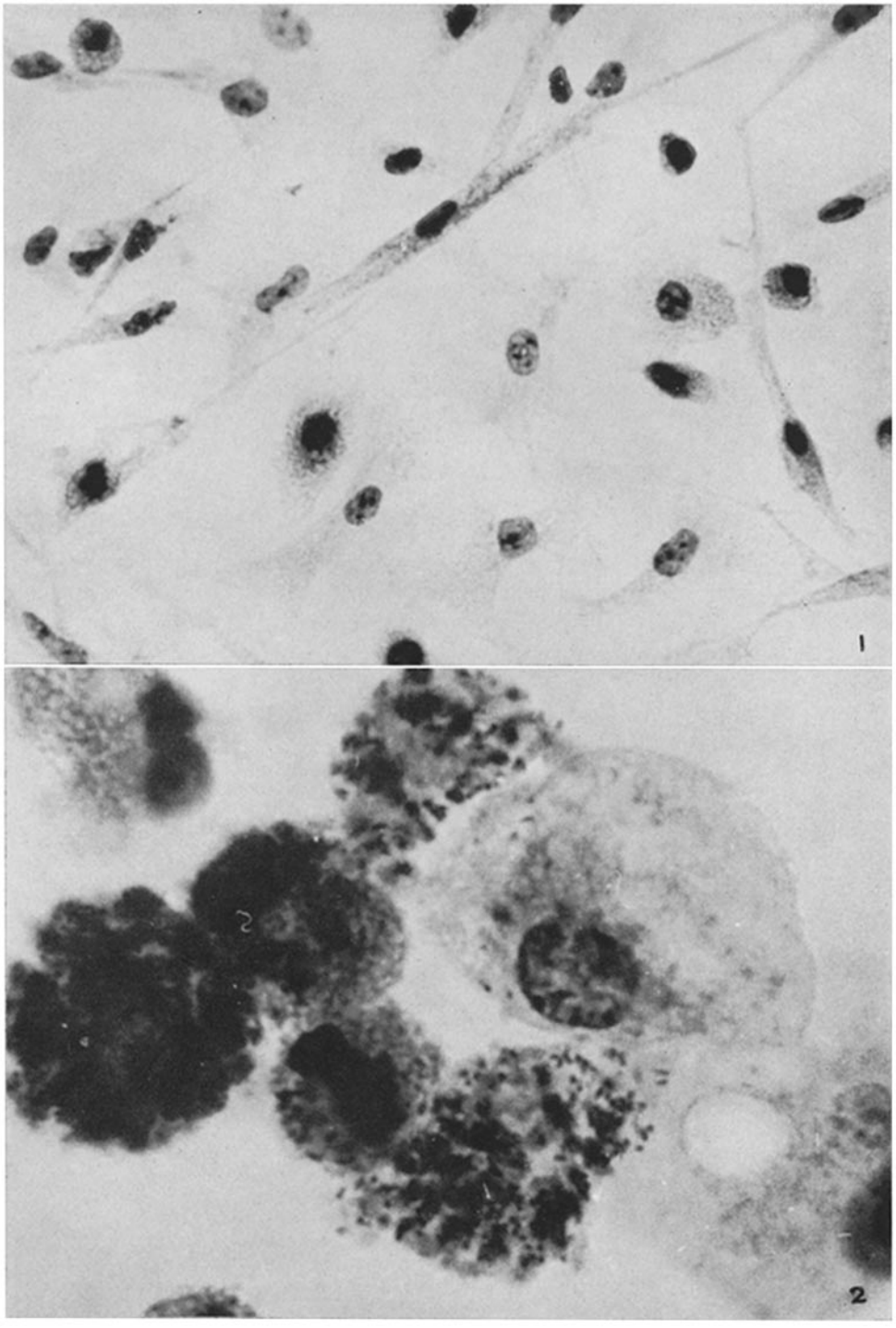
11. Braun, W., and Bonestell, A. E., Independent variation of characteristics in *Brucella abortus* variants and their detection, *Am. J. Vet. Research*, 1947, **8**, 386.
12. Henry, B. S., Dissociation in the genus *Brucella*, *J. Infect. Dis.*, 1933, **52**, 374.
13. Barski, G., Messore, G., and Lepine, P., L'exudat peritoneal artificiel source de cellules pour la culture des virus *in vitro*, *Ann. Inst. Pasteur*, 1955, **89**, 366.
14. Barski, G., Lamy, M., and Lepine, P., Culture de cellules trypsinees de rein de lapin et leur application a l'etude des virus du groupe herpetique, *Ann. Inst. Pasteur*, 1955, **89**, 415.
15. Perrin, T. L., Spontaneous and experimental encephalitozoon infection in laboratory animals, *Arch. Path.*, 1943, **36**, 559.
16. Nyka, K., Faherty, J. F., Malone, L. C., and Kiser, J. S., A histological study of the pathogenesis of tuberculosis in mice experimentally infected with bacilli of human type, *Exp. Med. and Surg.*, 1954, **12**, 367.
17. Jones, L. M., Further studies of the pathogenicity and immunogenicity of mucoid variants of *Brucella abortus* for guinea pigs, *J. Infect. Dis.*, 1953, **92**, 26.
18. Redfearn, M. S., Simon, E. M., and Berman, D. T., The establishment of colonial variants in guinea pigs infected with smooth *Brucella suis*, *J. Infect. Dis.*, 1956, **99**, 234.
19. Braun, W., Pomales-Lebrón, A., and Stinebring, W. R., Interactions between mononuclear phagocytes and *Brucella abortus* strains of different virulence, *Proc. Soc. Exp. Biol. and Med.*, 1958, **97**, 393.
20. Simon, E. M., Redfearn, M. S., and Berman, D. T., Competitive values of smooth and mucoid *Brucella abortus* in mixed infections in mice, *J. Infect. Dis.*, 1955, **96**, 268.
21. Braun, W., Studies on bacterial variation and selective environments: II. The effects of sera from *Brucella*-infected animals and from normal animals of different species upon the variation of *Brucella abortus*, *J. Bact.*, 1949, **58**, 299.
22. Dubos, R. J., and Schaedler, R. W., Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections. I. Protective effects, *J. Exp. Med.*, 1957, **106**, 703.
23. Schaedler, R. W., and Dubos, R. J., Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections. II. Enhancement of infection, *J. Exp. Med.*, 1957, **106**, 719.

EXPLANATION OF PLATES

PLATE 21

FIG. 1. Mouse peritoneal exudate cells 3 days after being placed in cell culture. Note the spindle-shaped cells among the more typical mononuclear phagocytes. × 400.

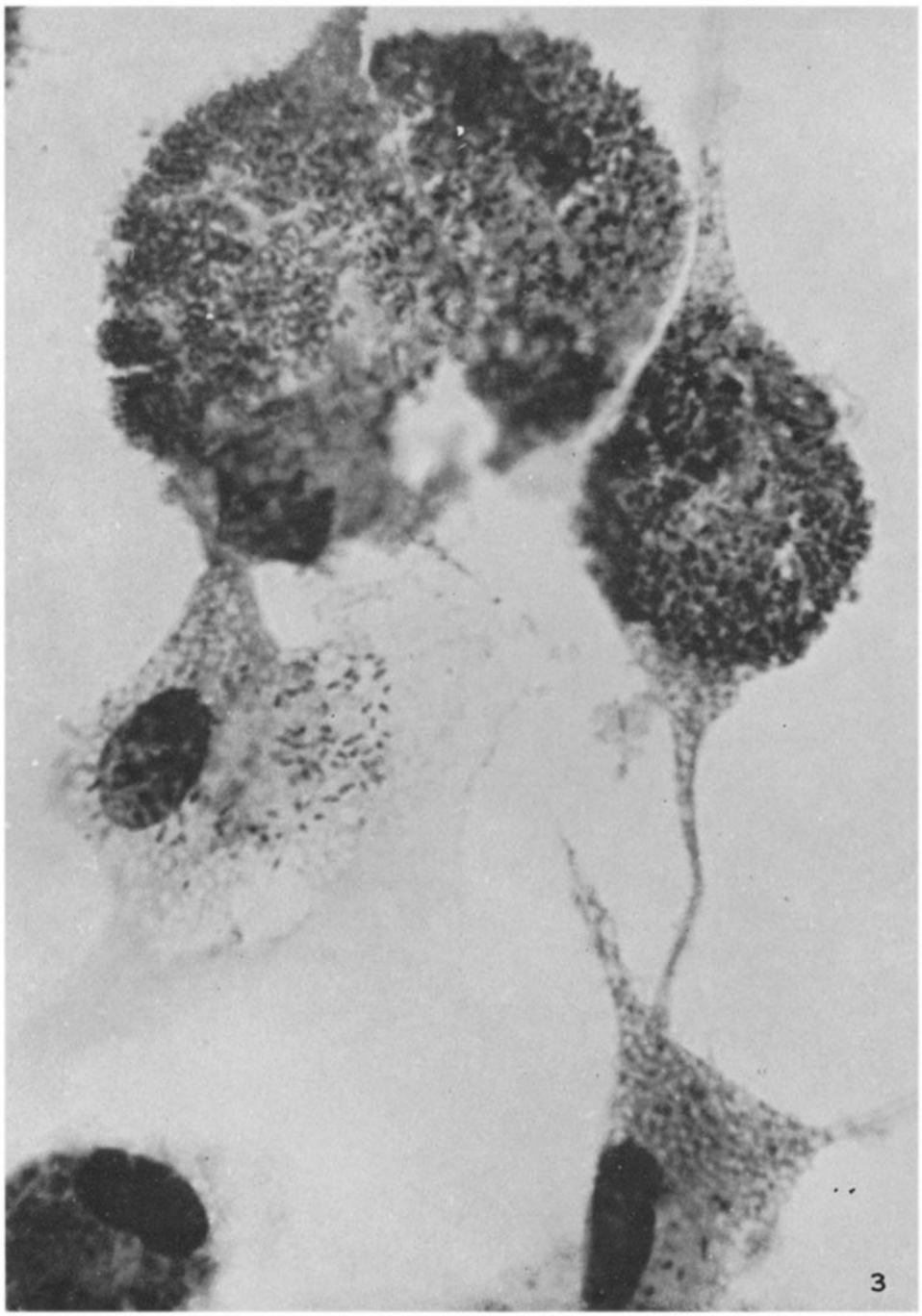
FIG. 2. Guinea pig monocytes 3 days after infection *in vitro* with smooth *B. suis*. Monocytes derived from normal guinea pig. × 1500.



(Holland and Pickett: Cellular basis of immunity in *Brucella* infection)

PLATE 22

FIG. 3. Mouse monocytes 3 days after infection *in vitro* with smooth *B. suis*. Monocytes derived from normal mice. × 1500.

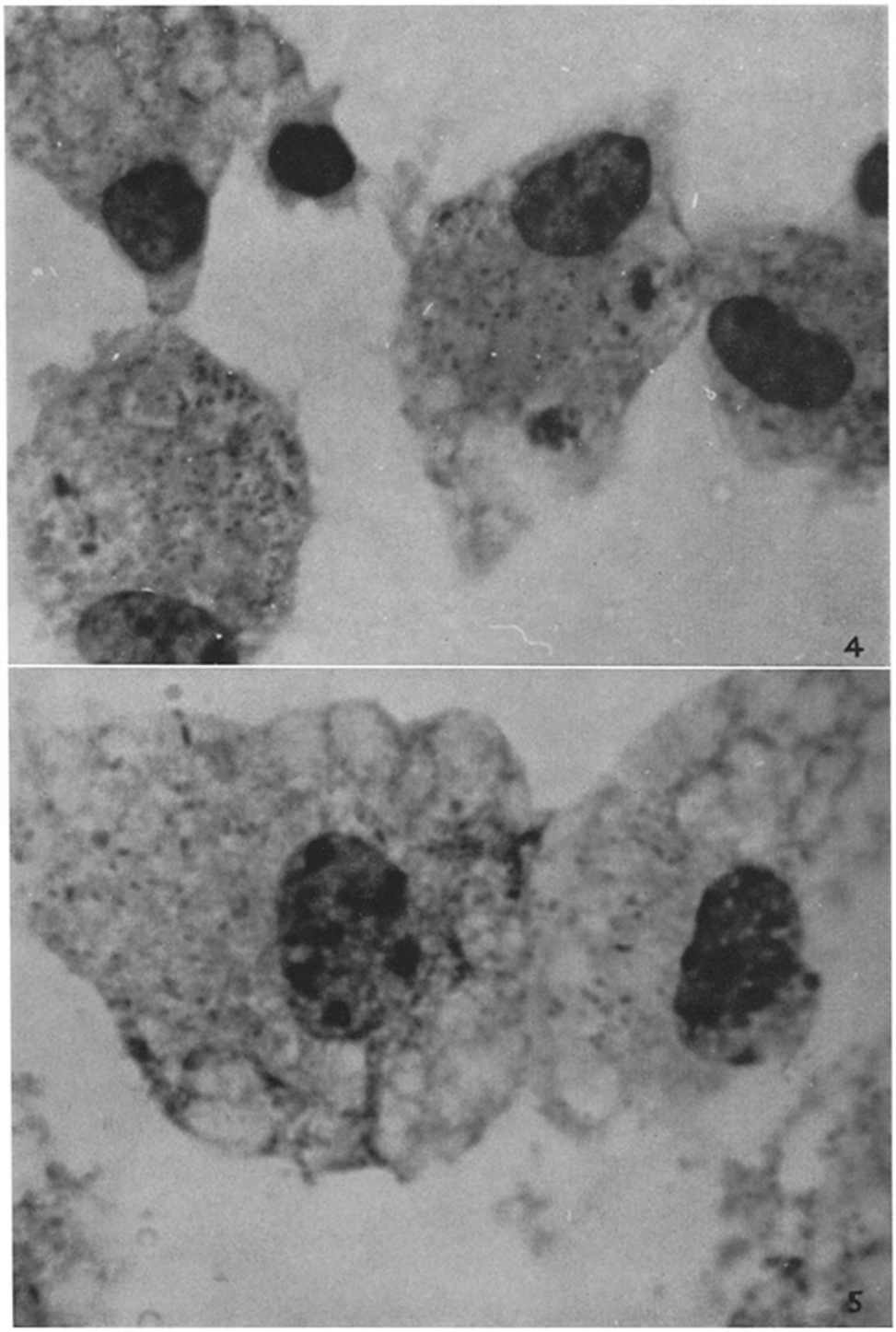


(Holland and Pickett: Cellular basis of immunity in *Brucella* infection)

PLATE 23

FIG. 4. Guinea pig monocytes 3 days after infection *in vitro* with smooth *B. suis*. Monocytes derived from immunized guinea pig. × 1500.

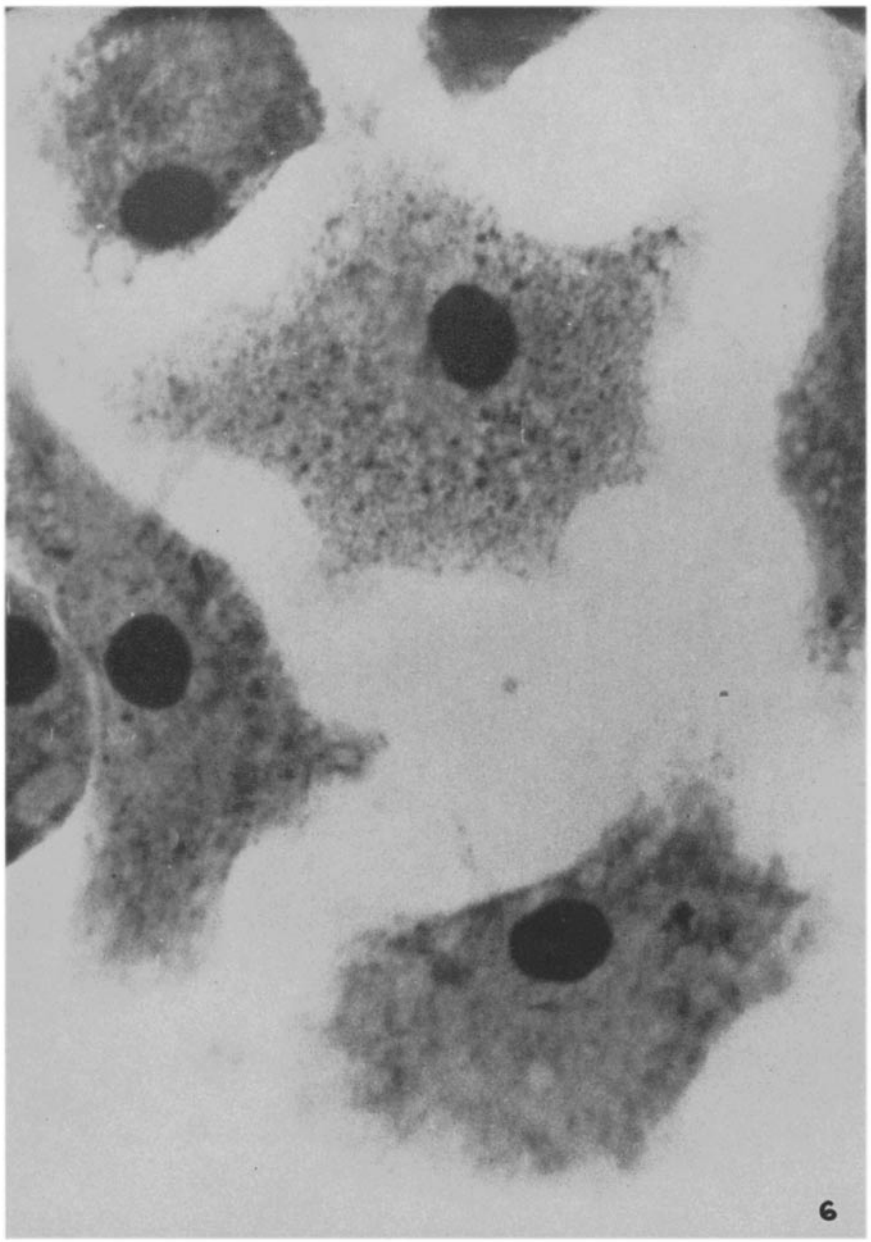
FIG. 5. Guinea pig monocytes 3 days after infection *in vitro* with smooth *B. suis*. Monocytes derived from immunized guinea pig. Different animal than in Fig. 4 × 1500.



(Holland and Pickett: Cellular basis of immunity in *Brucella* infection)

PLATE 24

FIG. 6. Mouse monocytes 3 days after infection *in vitro* with smooth *B. suis*. Monocytes derived from immunized mice. × 1500.



(Holland and Pickett: Cellular basis of immunity in *Brucella* infection)