JOURNAL OF BACTERIOLOGY
Vol. 88, No. 4, p. 1163-1168 October, 1964

MULTIPLICATION OF *BRUCELLA* IN CULTURED LYMPHOID AND NONLYMPHOID CELLS[1]

MARVIS RICHARDSON AND JANE N. HOLT

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan

Received for publication 19 June 1964

ABSTRACT

RICHARDSON, MARVIS (Michigan State University, East Lansing), AND JANE N. HOLT. Multiplication of *Brucella* in cultured lymphoid and nonlymphoid cells. J. Bacteriol. **88**:1163-1168. 1964.—Growth curves were established for the multiplication of *Brucella abortus* in cultured bovine cells. The number of viable brucellae was determined by colony count after lysis of the parasitized tissue cells. It was found that the number of brucellae dropped during the first 3 to 6 hr. This was followed by intracellular growth. Brucellae multiplied in uterine mucosal and fetal skin cells at an exponential rate with a 4-hr generation time. In contrast, only limited multiplication occurred in spleen cell cultures, usually approaching the stationary phase by 20 to 30 hr. Preliminary results indicated an average generation time of 8 hr in calf spleen cells. Differences were apparent in the ability of spleen cells from individual calves to support intracellular growth. This suggests that a relationship may exist between the establishment of intracellular pathogens in vitro and the natural resistance of the animal. By the use of fluorescein-labeled antisera, some insight was gained into the fate of brucellae in lymphoid cells. Fluorescent antisera stained intact brucellae and also revealed soluble antigen in the cytoplasm of reticularlike cells.

It is not known with any certainty whether the susceptibility to certain diseases depends upon the establishment of the etiological agent intracellularly. Culture of cells from naturally resistant and susceptible tissues presents an opportunity to examine this question. Intracellular brucellae are a common finding in brucellosis. In the pregnant infected cow, uterine and fetal epithelial cells completely filled with brucellae characterize the disease. Although cattle are the natural host for *Brucella abortus*, calves possess a high degree of resistance to infection; calves exposed to massive numbers of brucellae rapidly clear them from the spleen (Huddleson, 1942). With artificially infected cows, Payne (1959) showed that *B. abortus* localizes first in lymphoid tissue and then spreads to the uterus. Brucellae can be isolated from the bovine spleen soon after infection, but the organisms seldom localize in this organ. Doyle (1935) isolated *B. abortus* from only 10% of the spleens from 32 naturally infected cows. We found that *B. abortus* multiplies rapidly within cultured bovine uterine mucosal and fetal skin cells (Richardson, 1959; Richardson and Holt, 1962). In recent years, bovine spleen cells have been suspended with collagenase and grown routinely as a monolayer. This work was undertaken to compare the multiplication of *B. abortus* in spleen cells from calves and adult animals with that in fetal skin and adult uterine cells.

MATERIALS AND METHODS

Cell suspension and preparation of monolayer cultures. For the suspension of lymphoid cells, the method of Hinz and Syverton (1959) was followed with minor modifications. In brief, 2- to 4-mm pieces of fresh bovine spleen or lung were washed two times with GKN solution (sodium chloride, 0.8%; potassium chloride, 0.4%; glucose, 0.1%), placed in a screw-cap Erlenmeyer flask, and covered with 0.2% trypsin in GKN. The flask was flushed with 5% CO_2 in air, and the contents were agitated with a magnetic stirrer overnight at 4 C. The tissue was washed thoroughly with GKN solution and covered to a depth of 3 cm with 0.01% collagenase (Sigma Chemical Co., St. Louis, Mo.) in GKN. After incubation for 10 min in a water bath at 37 C, the tissue was agitated for 30 to 60 min in an incubator at 37 C. (Frequently, the tissue was treated a second time with collagenase, and the suspended cells were pooled with the first harvest. During this period, the cells from the first harvest were held in complete medium in a 5% CO_2 atmosphere.) The suspended cells were filtered through fine steel gauze, centrifuged for 10 min at 600 rev/min, and suspended in modified Eagle

[1] Journal Article No. 3301, Michigan Agricultural Experiment Station.

medium. The medium was prepared on the day of use with Minimum Essential Amino Acids and Vitamines (Microbiological Associates, Inc., Bethesda, Md.) and glutamine according to Eagle (1959) in the Saline F of Puck, Cieciura, and Robinson (1958); the complete medium contained 15% calf serum with no agglutinins for *Brucella*. Approximately 10^7 cells in 1 ml of antibiotic-free medium were grown as a monolayer in screw-cap tubes in an atmosphere of 5% CO_2 in air. The medium was renewed at 24 hr and at 48- to 72-hr intervals thereafter. Trypsinization of bovine fetal and adult tissues with 0.05% trypsin has been described (Richardson, 1959).

Brucellae. Typical, smooth, virulent strains of *B. abortus* grown for 24 hr were used. CO_2-dependent strains 3076 and 1359 were isolated from a human and from a cow, respectively, within the year prior to use.

Intracellular multiplication of brucellae. Tissue cells grown 5 to 8 days were exposed to 10^4 to 10^7 brucellae per tube for 5 hr. The brucellae-containing medium was then replaced with one containing 10 μg/ml of streptomycin. This was designated as zero hour. (Streptomycin at 10 μg/ml was found to kill brucellae rapidly in the external medium while apparently not interfering with intracellular multiplication of the organism.) To determine the number of intracellular brucellae at various times, the medium was removed and the tissue was washed with two 5-ml quantities of saline prior to cell lysis. The tissue cells were lysed by mechanical shaking for 1 hr with distilled water containing 0.05% Tryptose. Brucellae were enumerated by serial dilution and colony count. The geometric mean represented three or more tubes.

Fluorescent antibody staining. Two labeled antisera were used for direct staining: a commercially prepared anti-*B. abortus* rabbit serum labeled with fluorescein isothiocyanate (Difco); and anti-*Brucella* bovine serum conjugated with the same fluorochrome by the method of Riggs, Loh, and Eveland (1960). For indirect staining, an anti-*B. abortus* rabbit serum prepared in this laboratory was used in conjunction with a commercial anti-rabbit globulin labeled with fluorescein isothiocyanate (BBL). The tissue cells were washed with phosphate-buffered saline at pH 7.2, fixed with ethanol at 4 C for 30 min, and stained by the method of Riggs et al. (1958). For microscopy, a Zeiss GFL microscope with optovar attachment was used with an apochromat 40× oil immersion lens with iris diaphragm, an Osram HBO-200 lamp, primary BG 12/2 mm filter, and a OG-5 barrier filter.

RESULTS

Growth in lymphoid cells was studied first by determining the numbers of intracellular brucellae at 2 to 5 days. In lieu of adult animals of known history, eight young steers were selected to represent "normals." Steers are rarely vaccinated in calfhood and are seldom infected with *Brucella*. Spleen cells from five cows with serum agglutinin levels of 1:80 to 1:1,280 for *B. abortus* were tested also. Similar results were obtained with spleen cells from "normal" steers and infected cows. No marked or consistent difference was found between the number of brucellae at 0 hr and at day 2. With few exceptions, intracellular brucellae increased less than 1 log unit in this interval, and no increase occurred between days 2 to 5. (Several spleen cell cultures from normal and infected animals were grown in medium containing homologous or autologous anti-*Brucella* serum. The presence of antiserum, with or without complement during the intracellular growth period, did not affect the number of brucellae at day 2.) When the numbers were determined on day 1, fewer viable brucellae were found than at 0 hr. Examination of growth during the early hours of intracellular residence seemed indicated.

In our previous studies, growth curves had not been established for intracellular brucellae. Since multiplication in lymphoid cells was to be compared with that in nonlymphoid cells, growth curves were determined by use of both types of cells. Determination of the numbers of brucellae at short intervals after 0 hr revealed that the number dropped for several hours. For example, with a fetal skin cell culture which had been exposed to 10^6 brucellae per tube, the counts at 0, 1.5, and 3 hr were 41.0×10^3, 9.5×10^3, and 3.5×10^3, respectively. With cells exposed to 10^6 organisms, the counts were 55.0×10^4, 7.9×10^4, and 4.6×10^4 at these hours. In general, the number of brucellae dropped 1 to 1.5 log units. The low point occurred at 3 to 6 hr; thereafter, the numbers of intracellular brucellae increased. The initial drop occurred with cells from all of the tissues examined.

Distinct differences were found between the

extent of multiplication of *B. abortus* in fetal skin cells and that in calf spleen cells exposed to 10^6 brucellae (Fig. 1). Extensive work has proved that the growth curve presented is typical for fetal skin. After the initial drop in intracellular number, brucellae multiplied at a uniform rate through the 40-hr period. Exposure of cells to 10^3 to 10^7 brucellae resulted in a similar slope for intracellular growth. The generation time of 4 hr approximates that obtained with an optimal liquid medium. Brucellae multiplied at this rate in uterine mucosal and testicular cells from adult animals and in calf kidney cells (data not shown). The growth curves also revealed that what had been considered a prolonged lag phase, on the basis of similar numbers of brucellae at 0 hr and at 20 to 25 hr, resulted from a marked drop in initial numbers followed by exponential growth. The fetal cells used to obtain the growth curves (Fig. 1) originated from 5- to 6-month-old fetuses.

Multiplication in calf spleen cells was limited. Spleens 1 through 4 were from 12-week-old calves and spleen 5 was from a 6-week-old calf. As might be expected from the phagocytic nature of the population, calf spleen cells phagocytized more brucellae than did fetal skin cells. After 0 hr, the number of viable brucellae dropped less in the calf spleen cells than in the fetal skin cells. Calves 3 and 4 had received a subcutaneous injection of autoclaved tubercle bacilli 13 days previously, and were tuberculin-positive at the time of slaughter. Nonspecific antigen stimulation did not appear to affect the uptake or intracellular multiplication of brucellae. With calf spleen cells exposed to 10^6 brucellae, growth generally terminated at 18 to 25 hr, with an average generation time of 8 hr. Growth continued for a longer period in cells from one calf, at least 35 hr in cells from spleen 1, with a generation time of 9 hr. It seemed conceivable that exposure to large numbers of brucellae might overload phagocytic cells and limit multiplication. To examine this possibility, spleen cells from two 16-week-old calves were exposed to 10^4, 10^5, and 10^6 brucellae. For the sake of clarity, the curves obtained with 10^5 organisms have not been included, since results were similar. The number of brucellae associated with the cells at 0 hr was a direct function of the number used for exposure (Fig. 2). Brucellae multiplied to a somewhat greater extent and with a shorter generation time in the cells exposed to

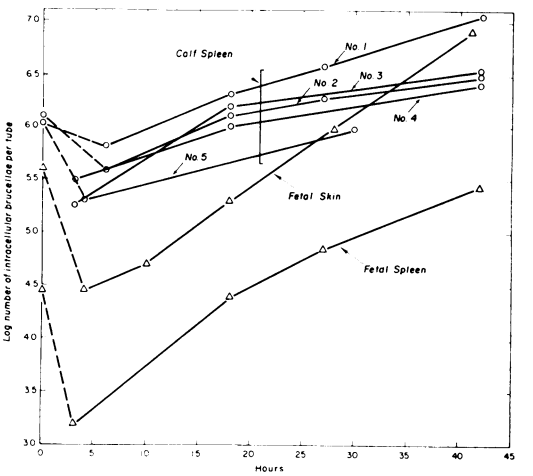

FIG. 1. *Growth of Brucella abortus in cells from fetal and immature animals. Cells were exposed to* 2×10^6 *brucellae.*

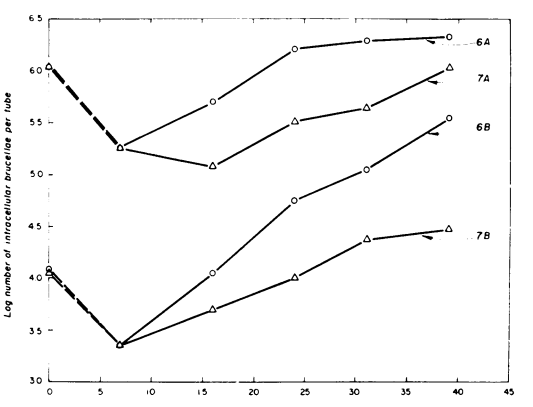

FIG. 2. *Effect of initial number of brucellae on growth in calf spleen cells. Cells A were exposed to* 2×10^6 *and cells B to* 2×10^4 *brucellae.*

10^4 organisms. More pronounced, though, was the consistently greater multiplication in cells from spleen 6 than in those from spleen 7, regardless of the numbers of brucellae phagocytized. The significance of the observation is unknown. Selection in cell type could have occurred in vitro. However, cells from spleens 6 and 7 were suspended on the same day and were carried through the test in parallel.

Growth curves were examined with spleen cells from adult animals, although the immunological status of most of the animals coming to slaughter was uncertain. Typical results are shown in Fig. 3.

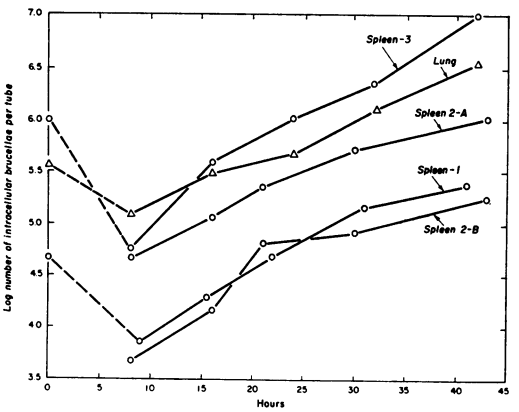

FIG. 3. *Growth of Brucella abortus in cells from mature animals. Spleen cells 2A and 3 and lung cells were exposed to 2 × 10⁶ brucellae; spleen cells 1 and 2B were exposed to 2 × 10⁵ brucellae.*

Spleen 1 came from a young, apparently healthy steer of unknown history. Spleen 2 was from a cow which had been vaccinated with *B. abortus* strain 19 in calfhood. This cow had been injected with *Staphylococcus aureus* via the udder for 6 months prior to slaughter and had shown systemic reactions. The supramammary lymph nodes were enlarged, and immunization doubtless affected the lymphoid cell population of the spleen. Cells from spleen 2A were exposed to 10^6 brucellae and cells 2B, to 10^5 brucellae. If the 21-hr point of 2B is ignored, the intracellular growth curves for brucellae in cells from this cow were similar to those obtained with cells from supposedly normal animals. Cells from spleen 3 originated from a "normal" steer, and had been subcultured for 4 months. Growth in these cells had not leveled off by 40 hr. Regretably, no cultures remained to determine whether growth continued at the same rate. Lung tissue contains a large lymphoid cell population. Growth in lung cells from six animals resembled that obtained with spleen cells. Usually a change in slope occurred in the 20- to 30-hr period. Generation time prior to the change of slope was from 5 to 8 hr. Because of the distinct differences in growth curves with cells from seven calf spleens, and because of the unknown history of most of the adult animals, no comparison of curves was attempted. Growth appeared to be limited more in cells from the calves than in cells from the adults.

With the use of fluorescein-labeled specific antisera (FA), some insight was gained into the fate of brucellae in lymphoid cells (Fig. 4). The particles in the macrophagelike cell in Fig. 4a corresponded in size and color to brucellae stained with FA. In addition to green organisms, gold-fluorescing material which might have been cellular debris phagocytized by the cell was evident. The round cell shown in Fig. 4b appeared completely filled with specific antigenic material. Little morphology could be discerned once macrophages were filled with brucellae. These cells resembled FA-stained cells in imprints prepared from supramammary lymph nodes from infected cows, if one allowed for distortion caused by separation from adjacent cells and by attachment to glass. In addition to those pictured, some cells contained dull particles in the cytoplasm which could have been disintegrating bacteria from which material was streaming. After exposure to *Brucella*, lung cell cultures from supposedly normal animals contained two types of cells containing specific antigen. Cells such as those in Fig. 4c contained uniform material which appeared to be soluble antigen. Only one end of the upper cell fluoresced brightly, and two discrete particles the size of brucellae were in this area. The second type of cell appeared to be a macrophage (Fig. 4d). Cells of both types were found in FA-stained imprints of lung tissue from infected cows. Some cultured spleen cells from naturally infected animals possessed nuclei which fluoresced intensely (unlike the negative nuclear images in the cells pictured) and nonspecifically. The cells with bright yellow nuclei were the size of small, medium, and large lymphocytes, often with specific, green particles the size of brucellae in the cytoplasm. Binucleate forms of the smallest cells were frequent. The cells adhered to the glass, they did not appear necrotic, and frequently they occurred in clusters as though coalesced or as a multinucleate cell.

DISCUSSION

Apparently, growth curves have not been established previously for intracellular bacteria. Because of the moderately long generation time, the multiplication of brucellae could be followed from the first hours of intracellular residence. These studies established intracellular growth curves of *B. abortus*. Findings presented revealed that a marked drop in viable brucellae preceded multiplication. The initial drop suggested an effect due to the antibiotics which were added at 0 hr to inhibit extracellular multiplication. Brucellae established within the cell at this time may have proceeded to multiply while those at the cell surface succumbed to antibiotic action.

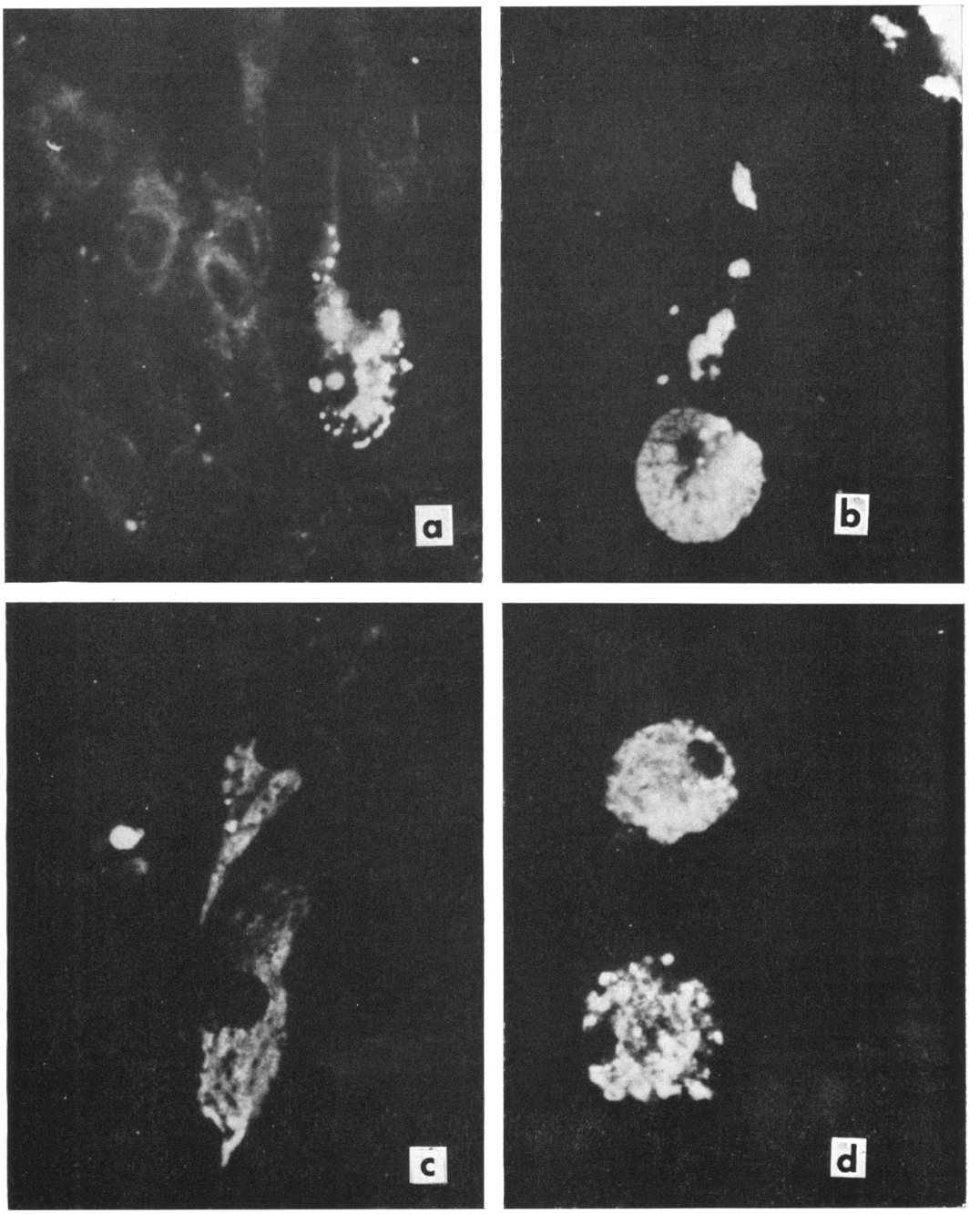

FIG. 4. *Spleen cells at 15 hr (a) and 3 days (b); lung cells at 3 days (c and d); direct FA staining.*

Cellular resistance also could account for the limited destruction of invading brucellae. In either case, proliferation within the cells could not be compared on the basis of the number of brucellae associated with the cells at 0 hr.

Marked differences were found in the growth curves of *B. abortus* in cells from nonlymphoid and lymphoid tissues. *B. abortus* parasitizes primarily uterine, placental, and fetal cells of the pregnant cow with brucellosis. In cells cultured from these tissues, brucellae multiplied exponentially. Microscopically, the progression of

events in fetal cells "infected in vitro" resembled that observed in the natural disease. The original infected cells ruptured, brucellae spread to adjacent cells, and disintegration increased until all tissue sloughed from the glass. Brucellae multiplied with equal rapidity in cultured testicular and kidney cells. Huddleson (1955) postulated that manifestation of brucellosis is triggered by some mechanism in the animal that permits the parasite to live at the expense of the epithelial cells. In one instance, the predilection for epithelial cells in a specific tissue was shown to rest on a chemical basis. Smith et al. (1962) found that erythritol, which is present only in fetal and placental tissues, is the cause of placental localization of *B. abortus* in bovine brucellosis. This carbohydrate enhances growth of *B. abortus*.

The data were inadequate for comparing the growth curves obtained with spleen cells from naturally resistant calves with those from somewhat more susceptible adult animals. Intracellular multiplication was difficult to assess with the use of fluorescent antibody, but breakdown to soluble antigenic material was definitely indicated. The growth curves of brucellae in spleen cells doubtless represent a summation of the multiplication and the enzymatic destruction of the parasite. Presumably, the spleen represents a first-line defense mechanism in the calf and adult bovine. Only limited multiplication appeared to occur in cultured spleen cells. On prolonged culture, spleen cells infected in vitro formed a sheet of fibroblastlike cells interspersed with occasional round cells. Areas of disintegration were not evident, although viable brucellae could be cultured from lysed cells for extended periods. One would anticipate latent infection in the spleen on the basis of these in vitro results. Cultured spleen cells might serve for the study of factors involved in transforming latent infections to overt ones.

Brucellae multiplied at different rates in spleen cells from some calves. This could be due to allelic differences in the animals or different stages of spleen development. The ability of spleen cells from individual animals to support growth consistently at different levels suggests that a relationship might exist between establishment of intracellular pathogens in vitro and natural resistance of the animal. Although calves are naturally resistant to brucellosis, a very small percentage of them become infected and remain so to maturity (Huddleson, 1942).

ACKNOWLEDGMENTS

The authors thank W. C. Eveland of the University of Michigan for assistance in the preparation of fluorescein-labeled antiserum.

This work was supported in part by grant G8829 from the National Science Foundation.

LITERATURE CITED

DOYLE, T. M. 1935. The distribution of *Brucella abortus* in the system of carrier cows. J. Comp. Pathol. Therap. **48**:192–195.

EAGLE, H. 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.

HINZ, R. W., AND J. T. SYVERTON. 1959. Mammalian cell cultures for study of influenza virus. I. Preparation of monolayer cultures with collagenase. Proc. Soc. Exptl. Biol. Med. **101**:19–22.

HUDDLESON, I. F. 1942. Immunity in brucellosis. Bacteriol. Rev. **6**:111–142.

HUDDLESON, I. F. 1955. Biochemical and histopathological reactions in the evaluation of bovine brucellosis. Mich. State Univ. Centennial Symp. Rept., p. 27–33.

PAYNE, J. M. 1959. The pathogenesis of experimental brucellosis in the pregnant cow. J. Pathol. Bacteriol. **78**:447–463.

PUCK, T. T., S. J. CIECIURA, AND A. ROBINSON. 1958. Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J. Exptl. Med. **108**:945–956.

RICHARDSON, M. 1959. Parasitization *in vitro* of bovine cells by *Brucella abortus*. J. Bacteriol. **78**:769–777.

RICHARDSON, M., AND J. N. HOLT. 1962. Synergistic action of streptomycin with other antibiotics on intracellular *Brucella abortus* in vitro. J. Bacteriol. **84**:638–646.

RIGGS, J. L., R. J. SIEWALD, J. H. BURCKHALTER, C. M. DOWNS, AND T. G. METCALF. 1958. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Am. J. Pathol. **34**:1081–1097.

RIGGS, J. L., P. C. LOH, AND W. C. EVELAND. 1960. A simple fractionation method for preparation of fluorescein-labeled gamma globulin. Proc. Soc. Exptl. Biol. Med. **105**: 655–658.

SMITH, H., A. E. WILLIAMS, J. H. PEARCE, J. KEPPIE, P. W. HARRIS-SMITH, R. B. FITZGEORGE, AND K. WITT. 1962. Foetal erythritol: a cause of the localization of *Brucella abortus* in bovine contagious abortion. Nature **193**: 47–49.