and P^{32} labeled phosphate into the cholesterol and phospholipids of plasma, liver, and aorta of chicks treated with Δ-4-cholestenone was measured. The response of the chick differed from that of the rat in that there was an enhancement of the inclusion of the radioactive labels into their respective products in plasma and liver. A significant decrease in aortic cholesterol content and specific activity was noted.

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Infection of Mice with G Variants (Micro-Colonies) of *Brucella abortus*. (24385)

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Hall and Spink(1) isolated a streptomycin-resistant strain of *Brucella abortus* in 1946 from blood of human patient with bacterial endocarditis due to *Br. abortus*, who had been treated with streptomycin. On agar plates this strain was composed mostly of minute colonies of brucellae having biochemical and serological characteristics of *Br. abortus*. These small colonies were markedly streptomycin-resistant, yet sublethal concentrations of the antibiotic stimulated growth, in marked contrast to the highly streptomycin-susceptible culture that had been obtained from blood of patient just prior to therapy with streptomycin. This small-colony strain was maintained in the laboratory for 7 years through many subcultures. Subsequently, Huddleson and Baltzer(2) in a study of the dissociation pattern of brucella isolated what they termed a "Type G (Micro-Colony Type) of *Brucella abortus*" from 10 of 12 strains of CO_2 dependent *Br. abortus*. They successfully infected guinea pigs with these micro-colonies, and postulated that these small forms of brucella might very well have been overlooked previously in material cultured from tissues and body fluids of infected animals and man. They emphasized that these micro-colonies were not the same as those described by Pickett and Nelson(3). Further studies in our laboratory were also concerned with the G forms of *Micrococcus pyogenes var. pyogenes*. Wise and Spink(4) reviewed the available information on micro-colonies originating from different species of bacteria, and pointed out that small-colony forms of staphylococcus could be readily selected out *in vitro* by several antibiotics. Evidence suggested this phenomenon also occurred in human patients who had been treated with antibiotics. Infections in animals were successfully established with G forms of staphylococci. The present report concerns investigations on brucella micro-colonies obtained *in vitro* from smooth strains of *Br. abortus* previously isolated from human patients. Growth characteristics, biochemical and serological properties, and *in vitro* susceptibility of these forms to the ac-

tion of antibiotics were studied. Infections with brucella G colonies were established in mice. Serologic studies were made; a search for tissue reactions due to brucellae were carried out; and duration of infection was ascertained.

Materials and methods. Isolation of micro-colonies from 14 smooth cultures of CO_2 dependent strains of *Br. abortus* obtained from human patients was attempted. The method slightly modified from that of Huddleson and Baltzer(2) was as follows: Cultures of *Br. abortus* were propagated on Albimi agar* slants for 48 hours in atmosphere of 10% CO_2. Bacterial growth was harvested in sterile saline in concentration of approximately 4 billion colonies/ml, and 0.5 ml of this suspension was spread over the surface of a tryptose agar† plate, incubated aerobically at 37°C for 12 days. Then with the aid of a stereoscopic microscope (20X magnification), small colonies were readily detected and transferred to the surface of another tryptose agar plate. A pure culture of G colonies was obtained and maintained by subculturing every 6 to 8 days. Before any specific study was carried out, 3 serial subcultures were made to ascertain colonial purity. Identification of micro-colonies as members of Brucella species was established by study of colonial appearance, by morphologic and staining reactions, and by bacteriostatic dye tests(5), using strains of *Br. abortus, Brucella suis* and *Brucella melitensis* as controls. Because of slow growth of G forms upon agar and lack of growth in broth further biochemical tests were not performed. More specifically the strains were studied by quantitative agglutination with specific anti-brucella rabbit serum. Two-fold serial dilutions of the antiserum were prepared in saline. Suspensions of parent and G strains were adjusted to the same turbidity through use of photoelectric colorimeter. Equal aliquots of cell suspensions were added to the serum dilution series as in a standard tube agglutination test(7). As described later, further confirmation of the identity of these strains was lent by production of specific anti-brucella agglutinins in mice. A modified tube-dilution technic was employed for determining *in vitro* susceptibility of micro-colonies to streptomycin and to oxytetracycline(6). A culture of G colonies grown on tryptose agar for 7 days was suspended in tryptose broth. Standardization of the inoculum was attempted by comparing turbidity of suspension of G colonies with a known suspension of parent cells. Normal-size aerobic colonies were freshly isolated from the CO_2-requiring parent organisms, that direct comparison of antibiotic sensitivity could be made without added CO_2. An inoculum of 5,000,000 organisms/ml (parent or G strain) was added to falling concentrations of antibiotic in tryptose broth. Aerobic incubation at 37°C was carried out. Because the G strains did not reproduce sufficiently to cause turbidity in the broth, a 72-hour bactericidal endpoint of antibiotic activity was determined by subculturing the contents of each tube on tryptose agar plates. Infections with G colonies were established in a population of 20 g ABC mice 2 to 3 months of age. A 9-day agar growth of G variants was suspended in tryptose broth, adjusted turbidimetrically, diluted, and injected intraperitoneally in 0.5 ml amounts. An equal inoculum of a 48-hour growth of parent strain was inoculated into a control group of mice. The titer of brucella agglutinins was determied on hearts' blood drawn at the time the animals were sacrificed for histological and bacteriological studies. A standardized antigen and technic was employed(7). The endpoint for agglutination test was the tube in highest dilution of serum that showed complete clumping of antigen and clear supernatant. An incomplete clearing of the suspended antigen was considered as "partial" agglutination titer of serum. The survival of G colonies in animals was determined by grinding up the spleen under sterile conditions with 0.7 ml of saline, and inoculating 0.2 to 0.6 ml on the surface of each of 3 plates of tryptose agar. Aerobic incubation was carried out, the colonies counted and identified.

Results. Morphology and growth properties of G colonies. Small colonies were successfully isolated and subcultured from 11 of 14 cultures of *Br. abortus*. G colonies were

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† Difco Labs., Detroit, Mich.

isolated with ease from 6 of the 14 cultures. Isolates were successful with 4 additional strains following a second attempt. A few small colonies were isolated from the eleventh strain in the first attempt, but no growth was obtained with subsequent transfers. No small colonies could be isolated from 2 cultures, both obtained originally from the same patient. The micro-colonies on the surface of tryptose agar plates were approximately 0.1 mm in diameter, otherwise they had the same gross appearance as the large parent colonies. Morphological and tinctorial properties of the 2 types of colonies when examined with Gram's stain were the same. Whereas growth of parent large colonies was readily supported on Albimi agar plates, growth of transplanted micro-colonies did not take place on this medium. Good growth occurred on the surface of tryptose agar. Both media had the same content of agar, but the surface of the tryptose agar plates was softer. Viable cells from micro-colonies persisted in tryptose broth, but little or no reproduction took place.

After the G forms had been transferred several times on tryptose agar, a few large colonies made their appearance under aerobic conditions. When the tryptose agar plates were placed in an atmosphere of 10% CO_2 reversion to the larger colonies readily occurred with every culture. When G cultures were placed in tryptose broth and 10% CO_2 was added, the strains again reverted, and luxuriant growth appeared. Upon subsequent subculture to tryptose agar plates, large colony forms predominated.

Attempts to isolate G colonies from broth suspensions of large colonies of the 3 species of brucella exposed to varying concentrations of antibiotics were unsuccessful. This method for isolating small colonies had proved successful for the staphylococcus. G colonies of brucella were not recovered from cultures of large colonies grown on the surface of gradient agar plates containing antibiotics(8).

Bacteriostatic action of dyes on micro-colonies. Three different cultures of G colonies along with parent strains were subjected to bacteriostatic action of basic fuchsin and to thionin, using tryptose agar as the nutrient base. Growth of parent cultures of *Br. abortus* was characteristically inhibited by thionin, but growth occurred in the presence of basic fuchsin. The results with G strains were not so definitive in the dye concentrations ordinarily employed. None of the G variants grew on thionin (1/100,000) in accordance with the behavior of large colony *Br. abortus* strains; however, the G strain also grew with difficulty upon 1/80,000 basic fuchsin dye plates.

Agglutination of G cultures with anti-brucella serum. All of the 11 cultures of micro-colonies were readily agglutinated with anti-brucella rabbit serum, the endpoint of suspensions being the same for small and large parent cultures.

Sensitivity of G cultures to antibiotics. Sensitivity tests were carried out with streptomycin and oxytetracycline, using 10 cultures of G colonies and large colony aerobic variants of the 10 parent strains. With streptomycin the average bactericidal endpoint of the 10 parent strains was 1.3 μg/ml while that of corresponding G strains was 2.15 μg/ml, a difference of questionable significance. With oxytetracycline the parent strains were killed by an average concentration of 50 μg/ml while the G strains were not inactivated until an average concentration of 150 μg/ml was reached. The difference in antibiotic concentration between tubes was 2-fold.

Serological and bacteriological studies in mice following infection with G colonies. Heavy broth suspensions of 3 different G cultures were prepared, and 0.5 ml of each strain, or approximately 2.5 billion organisms, was injected intraperitoneally into a pair of mice. The mice were observed for 21 days, then sacrificed by withdrawing blood from the heart. During the period of observation the mice exhibited no ill effects. Post-mortem examination revealed no gross abnormalities in liver, spleen or kidneys. Agglutination and bacteriological studies showed that 5 of the 6 mice had been successfully infected. In 5 infected mice, agglutinins were demonstrated in the serum. Two mice had a brucella agglutinin titer of 1 to 80; 2 had a titer of 1 to 320, and one, a titer of 1 to 1280. G colonies were successfully isolated from the emulsified spleens of 3 of the 6 mice. G colonies were

TABLE I. Serological and Bacteriological Studies on Mice Infected with G Colonies of *Br. abortus* and Sacrificed at Varying Times after Infection.

Strain	2 mo after infection		5 mo after infection		7 mo after infection	
	Reciprocal of agglutination titer	Colonies of brucella	Reciprocal of agglutination titer	Colonies of brucella	Reciprocal of agglutination titer	Colonies of brucella
524	80	0	40 (partial)	0	40 (partial)	0
	80 (partial)	0	80 "	0	20 "	0
552	80 "	0	40 "	0		0
	80 "	21/0.2 ml	20 "	0	40	0
1302	40 "	1/0.2 ml	80 "	0	40	0
	80 "	1/0.6 ml	40	0	40 (partial)	0
2121	40	0	40 (partial)	0	40	0
	320	8/0.6 ml	20 "	0	40	0
2165	80 (partial)	0	40 "	0	20	0
	40 "	0	20 "	0	40 (partial)	0

not grown from spleens of 2 mice, but when emulsions were incubated in the presence of CO_2, larger brucella colonies were obtained. These observations demonstrated that mice could be infected successfully with G cultures, and the organisms could be recovered from spleen within 21 days after infection.

A second experiment was carried out to determine the relative pathogenicity of the parent strain with that of the G variant and also to determine how long G colonies could be recovered after infection from spleens of infected mice. All organs were examined both grossly and microscopically. Large colonies of 5 different parent strains of *Br. abortus* and corresponding G isolates were employed. Approximately 200,000,000 organisms of each strain were injected intraperitoneally into groups of 6 mice (a total of 60 mice). At the end of 3 weeks all mice injected with the parent large colony strains were dead. Mice injected with the G variants displayed no signs of illness, and gained weight until sacrificed. Two mice of each strain were sacrificed 2 months after infection; another pair 5 months after infection; and the last pair 7 months after infection. The results of the agglutination reaction with sera and the bacteriological studies with emulsified spleens are presented in Table I. Brucella agglutinins were demonstrated in serum of all but one of the 30 mice, indicating that infection had taken place. G colonies were isolated from spleens of 4 out of 10 mice 2 months after the organisms had been injected. However, no G colonies could be cultured from the spleen 5 and 7 months after the mice had been infected. Microscopic examination of sections of the liver, spleen and kidneys stained with hematoxylin and eosin did not reveal lesions compatible with a brucella infection in the mice sacrificed 2 and 5 months after being infected.

Summary. The foregoing data show that pure cultures of micro-colonies can be isolated readily from smooth strains of *Br. abortus*. These G cultures can be maintained for several generations if subcultures are incubated aerobically. When fresh transplants are incubated in an atmosphere of 10% CO_2 reversion to the large colonial form occurs. Mice can also be easily infected with G colonies and the small forms can be recovered from the tissues, though the infection is of low pathogenicity and of relatively brief duration. The practical significance of these observations in animal and in human brucellosis under natural conditions cannot be ascertained at this time. Huddleson and Baltzer(2) did report the presence of G colonies of *Br. abortus* in a culture of stomach contents of an aborted bovine fetus, and Hall and Spink(1) isolated *Br. abortus* G colonies from a human patient. On several occasions we observed the presence of small colonies in cultures of *Br. melitensis* isolated from tissues of mice. It is suggested that a more intensive study should be made of fluids and tissues of animals and man for presence of small colonial forms of brucellae.

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Influence of Cortisone on Infection in Mice Caused by Strain 19 *Brucella abortus.* (24386)

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When mice were pretreated with cortisone and then infected with a smooth culture of *Brucella suis,* a fulminating brucella infection resulted(1). Hepatic abscesses teeming with brucellae were observed in the cortisone-treated mice, in marked contrast to occasional granulomatous lesions seen in saline-treated control mice. Since cortisone intensified brucella infection due to an invasive strain of brucella, it was important to determine if the steroid accelerated infection due to a brucella strain with attenuated virulence. Strain 19, *Brucella abortus* is a stable, smooth dissociant of *Br. abortus,* widely used for immunizing cattle against brucellosis. This strain does not require additional CO_2 for growth. It is doubtful whether a persistent infection can be established in animals with this strain. There is no evidence that infection with strain 19 has been transmitted from animal to animal or that man has acquired the disease from animals immunized with the vaccine. However, there are several well documented cases of human brucellosis caused by strain 19, in which man became accidentally infected while immunizing cattle(2).

Materials and methods. Adult white male ABC mice weighing approximately 20 g were used. Twenty mice were injected daily subcutaneously with 0.2 ml saline, and 20 mice were injected daily for 13 days with 0.5 mg of cortisone acetate. Each of these 2 groups were injected intraperitoneally on the third day of the foregoing treatment with 10 million organisms of strain 19 *Br. abortus.* Ten uninfected control mice were treated daily with 0.5 cortisone for 13 days. The 2 groups of infected mice were sacrificed 7, 15 and 21 days after infection. The titer of brucella agglutinins was determined on heart blood. Liver, spleen, and kidney of each mouse were examined grossly, and quantitative brucella cultures of the emulsified organs were made on Albimi brucella agar.* Sections of liver were stained with hematoxylin and eosin and examined microscopically.

Results. Saline-treated infected mice appeared healthy throughout period of observation, whereas cortisone-treated infected and uninfected mice lost weight. Serological and bacteriological studies on the saline-treated and cortisone-treated groups of infected mice are presented in Table I. Cortisone did not interfere with formation of brucella agglutinins, the titers being approximately the same in the 2 groups. There was a significantly greater number of brucella colonies in the organs of the cortisone-treated mice. In contrast with previous studies on effects of cortisone on tissues invaded by the more virulent species of Brucella, there was no outstanding difference between saline-treated and cortisone-treated mice infected with strain 19. Seven days after infection the saline-treated group showed a large number of well-defined

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