Intracellular Behavior of Brucella Variants in Chick Embryo Cells in Tissue Culture.* (22792)

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Ability of smooth brucellae to localize intracellularly is well known. Brucellae within leucocytes are protected *in vitro* against high concentrations of antibiotics and antiserum for several days(1,2). It has recently been reported, however, that monocytes derived from immune animals restrict intracellular multiplication *in vitro*(3). Non-smooth variants of *Brucella* have not been studied with regard to intracellular residence, although the selective effects of metabolite accumulation and lowered oxygen tension *in vitro* have been thoroughly investigated(4-9). Suppressive effect of selective serum (SS) factor on non-smooth brucellae *in vitro* has also been extensively studied(10). Non-smooth brucellae do not persist long *in vivo* except under conditions where selective metabolites may accumulate, such as in abscessed tissue or aborted fetal material(11-13). Thus, they have been generally considered to be of little significance in brucellosis. However, several instances of mutation from smooth to mucoid *in vivo* have recently been demonstrated, in which the mucoid variants were recovered as often from unabscessed as from abscessed tissue(14).

The present study is concerned with the selective effect of an intracellular environment upon smooth and non-smooth variants of *Brucella*.

Materials and methods. Tissue culture. The culture vessels were 16 x 125 mm screw-cap test tubes coated on the inside with a thin film of formvar (polyvinyl formal) according to the method of Barski *et al.*(15). Each culture tube contained 1.5 ml of medium consisting of 20% fresh rabbit serum, 20% chick embryo extract (1/1) and 60% Hanks' balanced salt solution. Ten or 11-day chick embryos (with head, legs and wings removed) were aseptically ground to a fine liquid pulp, and approximately 0.01 ml of this tissue was placed into each culture tube. The tubes were incubated at 37°C in an almost horizontal stationary position. Within 24 hours a heavy growth of fibroblasts covered the lower walls of the tubes. The medium was renewed every 2 or 3 days. *Bacterial cultures.* Brucellae were grown and maintained on *Brucella* agar (Albimi), enteric bacilli on MacConkey agar (Difco) and streptococci on 5% sheep blood agar. The smooth strains of *Brucella* used were *Brucella abortus* a77, a5 (CO_2 dependent) and strain 19 (Lederle no. 1952a); *Brucella suis* s101, s99 and s25, and *Brucella melitensis* m62. I (intermediate), R (rough), M (mucoid) and S^R (smooth-rough) variants of each species were obtained by prolonged growth in *Brucella* broth (Albimi) containing 300 μg/ml DL-α alanine. The acriflavin test(6) was used in conjunction with anti-S antiserum and with colonial observation(4) for differentiation of variants. The streptococcus used was a group A, β hemolytic *Streptococcus pyogenes* freshly isolated following four consecutive passages in mice. The enteric bacilli were laboratory strains of *Escherichia coli, Alcaligenes fecalis* and *Salmonella typhosa. Infection of tissue cultures.* The fibroblasts were infected on second day of culture by addition of approximately 10^6 bacteria to the tissue culture medium. Five hours later streptomycin was added to a final concentration of 10 μg/ml to kill extracellular bacteria and to prevent further extracellular growth in the medium. For the streptococcal work 1 unit/ml of penicillin was substituted for streptomycin. For the faster-growing streptococcus and enteric bacilli an infection period of only 2 hours was allowed before addition of antibiotic. At daily intervals after cell infection, a roughly quantitative determination of viable bacteria within the cells was made by scraping a platinum loopful (1/200 ml) of infected tissue from walls of tube and streaking it evenly

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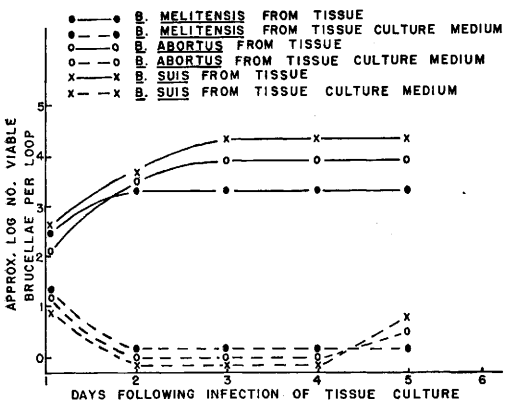

FIG. 1. Three typical experiments showing viable brucellae recovered from infected tissue as compared to those recovered from tissue culture medium in the same tube. The few bacteria appearing in the medium about the fifth day are due to degeneration of some of the infected cells.

on agar plate. A loopful of medium from the same tube was also plated out as a control against presence of extracellular bacteria.

Results. Smooth strains of all 3 species of *Brucella* entered the fibroblasts and not only survived but multiplied therein as shown in Fig. 1. If, however, the tissue cultures were heat-killed (56°C—1 min) either before or after infection, the cells were no longer capable of protecting the brucellae from the streptomycin, and no viable brucellae could be recovered after 30 hours. Thus, under the conditions of the experiment all viable bacteria recovered from tissue cultures represent growth or survival within living avian cells. When formvar-coated cover slips were included in the culture tubes, then withdrawn at intervals and stained by Perrins' modification of the Goodpasteur stain(16), intracytoplasmic bacteria were seen within a small percentage of the fibroblasts. Some fibroblasts contained only a few bacteria after several days infection, while others were nearly filled. To see whether this ability to grow within fibroblasts is unique to brucellae, several other bacterial species were studied under the same conditions. Fig. 2 shows that although all 5 species were phagocytized and thereby survived for a day or two, only the 2 intracellular species *S. typhosa* and *B. suis* multiplied or survived for long.

Prolonged culture of Brucella-infected tissue. Tissue cultures infected with brucellae did not seem to be grossly damaged by this infection. Therefore, prolonged subculture of infected fibroblasts was attempted to determine whether the host cells or the parasite would ultimately prevail over the other. In this experiment the infected fibroblasts were transferred to new tubes every 4 days following trypsinization and washing. Fibroblasts infected with *B. melitensis* m62 and *B. abortus* a5 and a77 were maintained through 7 transfers for 32 days and were still moderately infected when the subcultures were discontinued. Apparently only lightly infected and uninfected fibroblasts survived each trypsinization, since the number of viable brucellae recovered dropped following each transfer, then increased again before the next transfer. Fibroblasts infected with *B. suis* s101 became very granular after 4 to 6 days of infection, became heavily vacuolated and did not survive the second or third trypsinization. The slower growing *B. suis* strains s25 and s99 also eventually destroyed the fibroblasts they infected, although more slowly (after 3 or 4 transfers or 12 to 16 days). Over 10^4 viable brucellae per loop were recovered from cells showing cytopathogenicity due to each of the 3 B. *suis* strains. Prolonged intracellular passage of all 3 species enhanced their colonial smoothness. The colonies recovered developed

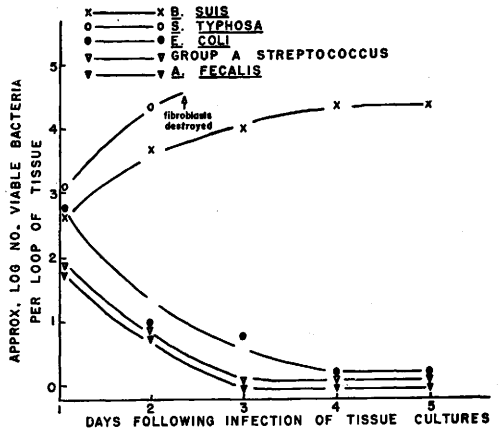

FIG. 2. Comparison of intracellular survival of several bacterial species. *S. typhosa* grew so rapidly intracellularly that cytopathogenicity occurred and streptomycin resistant mutants appeared in about 2 or 3 days.

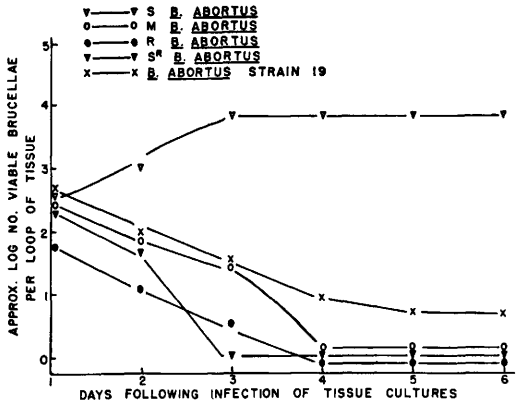

FIG. 3. Intracellular growth of smooth brucellae as compared to non-smooth variants and strain 19 in separate tissue cultures.

more slowly, were less granular and more transparent and bluish.

Intracellular behavior of non-smooth brucellae. M, R, and S^R variants from each of the 3 species were examined for their ability to grow intracellularly as did the smooth parent strains. Strain 19 was also tested. The results of a typical experiment are seen in Fig. 3. It is obvious that neither the non-smooth variants nor strain 19 multiplied or remained viable for long intracellularly. Similar results were obtained with non-smooth variants of *B. melitensis* and *B. suis*. Cells were also infected with mixed inocula of smooth and non-smooth types in various proportions. In all cases the smooth type showed a marked selective advantage (Fig. 4). In another experiment the slimy sediment from a thirty day broth culture of *B. melitensis* was used as an inoculum, and although only non-smooth variants were present on the first 3 days, smooth variants predominated on the fourth day after infection. We next attempted to obtain dissociation from non-smooth to smooth in tissue culture, followed by selection of the smooth type. Therefore, very heavy inocula of non-smooth variants were used to increase the probability of obtaining the S mutant. In some cases this was successful. S colonies were quite often obtained from I inocula and more rarely from M inocula, but never from R or S^R inocula. However, it was observed that very large inocula (over 10^7) of even non-smooth variants caused overwhelming infection, often leading to cytopathogenicity and even to prolonged survival of non-smooth types within the fibroblasts.

Effect of Brucella antiserum and of α alanine in tissue culture medium. It was felt that the presence of the SS factor(10) in the normal rabbit serum of the tissue culture medium might have influenced the results. Therefore, fresh antiserum from rabbits infected with *B. melitensis* was substituted for normal serum. Despite the presence of anti-S antibodies and complement, and despite the absence of the SS factor, the results were not altered. The S type still multiplied intracellularly and the non-S variants were eliminated. Likewise, the addition of DL-α alanine to the tissue culture medium in a final concentration of 300 μg/ml [sufficient to cause rapid selection of non-smooth variants in liquid culture(17)] did not alter the smooth selective character of the intracellular environment.

Discussion. It is apparent that brucellae and other bacteria capable of intracellular growth are in some way resistant to those intracellular mechanisms which inhibit other bacteria such as *E. coli* or the streptococcus. Possibly the loss of surface antigens(18) accompanying mutation from S to non-S might help explain the increased vulnerability of non-smooth brucellae to cellular defenses.

The survival of *Brucella* for long periods

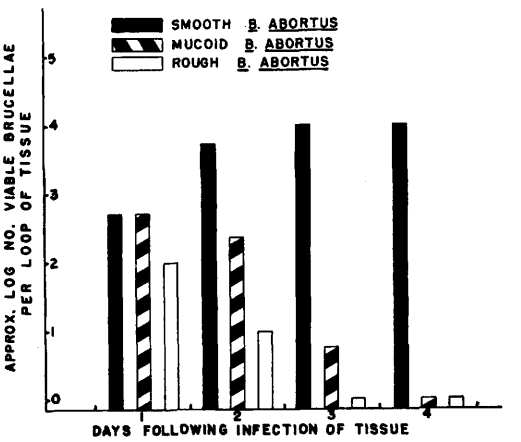

FIG. 4. Intracellular selection of the smooth type in a typical tissue culture infected with a mixed inoculum containing approximately 10^6 each of S, M, and R variants.

within fibroblasts cultured in a bactericidal medium, without gross destruction of the tissue, helps explain the capacity of *Brucella* to cause insidious, chronic disease. This is in contrast to *S. typhosa* which also multiplied intracellularly but which caused rapid cytopathogenicity.

The unaltered smooth selective capacity of fibroblasts cultured in antiserum (lacking in SS factor), indicates one reason why animals lacking the SS factor, and even immune animals, clear non-smooth brucellae from their body much more rapidly than they do the smooth type(14).

It appears that the virulence of *Brucella* is correlated with their ability to multiply intracellularly since the avirulent non-smooth variants and strain 19 are unable to do so. Further work is in progress on the intracellular infection of mammalian cells derived from normal and immune animals.

Summary. 1) Smooth brucellae were ingested by, and multiplied within, fibroblasts in tissue culture. Non-smooth variants and strain 19 neither multiplied nor survived long intracellularly unless the cells were massively infected. Brucellae remained viable intracellularly for over 30 days despite the presence of a bactericidal concentration of streptomycin in the tissue culture medium. Extensive cytopathogenicity was not evident except with *Brucella suis* which caused slow destruction of the fibroblasts. Under the same conditions of culture *Salmonella typhosa* multiplied rapidly intracellularly and caused complete cell destruction within several days. 2) Mixed infections of fibroblasts with smooth and non-smooth brucellae showed the intracellular environment to be highly selective for the smooth type. Mutation from I to S and M to S, followed by selection of the S type, occurred in several heavily inoculated tissue cultures. The addition of anti-S antiserum or DL-α alanine to the tissue culture medium did not affect the smooth-selective behavior of the intracellular environment.

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