Continuous Grouth of *Brucella abortus* in Mononuclear Phagocytes of Rats and Guinea Pigs.* (24962)

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In naturally acquired bacterial infections, infecting organisms tend to be passed from host to host without growth on lifeless media. This may at least in part contribute to the maintenance and production of proper antigenic and physiologic states required for establishment of the parasite in the new host. In studying factors of major importance in pathogenesis and control of naturally acquired brucellosis, it would therefore appear likely that, in comparison with organisms grown on lifeless media, *Brucella abortus* organisms grown intracellularly might better approximate the state of organisms as ordinarily transmitted. Recent studies(1-4) have demonstrated that such intracellular growth can be accomplished under *in vitro* conditions by permitting brucellae to multiply with mononuclear phagocytes maintained in tissue culture. However, all prior studies have been concerned with only one cycle of cultivation under these conditions. This may not have sufficed to produce those physiologic and genetic states of importance in natural disease. Therefore, this study was undertaken to determine if continuous passage of *Br. abortus* in mammalian mononuclear cells is possible. Results indicate that such continuous passage without intervening cultivation on lifeless media can indeed be accomplished. In addition, it has been observed that mammalian species supplying host cells significantly affects the outcome of such continuous intracellular cultivation.

Methods. Br. abortus, strain SA-S, isolated long ago from a human case in Puerto Rico, and its rough variant SA-R(3) were studied. Guinea pig and rat mononuclear cell cultures were prepared according to method of Pomales-Lebrón and Stinebring(1). Normal, young, male guinea pigs of the Hartley strain and normal adult female white rats provided the mononuclear cells. For harvesting these cells, the peritoneal cavity of animal was washed with Hanks' solution 2 days after intraperitoneal injection of 20 ml of saline. Approximately 1 x 10^7 of washed mononuclear cells were mixed with approximately 2.5 x 10^8 brucellae in 5 ml of 30% autologous serum-Hanks' medium. The mixture was pipetted into a T-30 flask and incubated 2 hours. During this time, mononuclear cells containing ingested brucellae attached themselves to the floor of flask. The supernate was then replaced with identical medium supplemented with 10 μg of streptomycin/ml. This concentration of antibiotic has been proved capable of killing extracellular brucellae but not intracellular organisms. Three days later cultures were gently washed twice with warm Hanks' solution to remove residual streptomycin, small glass beads† and 8 ml of Hanks' solution were added, and the flasks shaken with rotating motion of the hand. This procedure disrupted the cells without affecting the brucellae. A portion (3.5 ml) of resulting supernate containing the bacterial harvest was immediately added to 1.5 ml of a fresh monocyte-serum mixture, thus initiating the next cell culture. Viable counts on aliquots of harvest were done on tryptose agar.

Results. Continuous transfer of Br. abortus in guinea pig cells. The data in Fig. 1 and Table I demonstrate that it was possible to transfer smooth organisms of *Br. abortus* through monocytes for more than 20 transfers without intervening growth on lifeless media. As far as yield of viable bacteria/monocyte culture was concerned, there was an initial period of continually increasing viable count, followed by period of relative stability. Approximately 10^8 organisms/culture appeared the maximum yield. Poor harvests were obtained whenever microscopic observation in-

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† Minnesota Mining and Manufacturing Co. 3M Superbrite No. 100.

TABLE I. Harvest (per Flask) of *Brucella abortus* Passaged in Continuous Transfer.

Passage	SA-S, guinea pig cells	SA-S, rat cells	SA-R, guinea pig cells
0	1.7×10^6	2.0×10^6	5.2×10^7
1	2.5 "	5.2 "	6.0×10^6
2	6.0 "	1.8×10^7	4.0×10^3
3	2.2×10^7	9.6×10^6	$<1.0 \times 10^2$
4	6.8 "	2.4 "	Discontinued
5	1.9×10^8	1.0 "	
6	2.7 "	3.6×10^5	
7	1.6 "	1.1×10^6	
8	1.2 "	2.5×10^5	
9	1.8 "	1.2 "	
10	6.8×10^7	4.8×10^3	
11	2.2 "	2.3 "	
12	5.2 "	2.0 "	
13	6.4 "	1.0 "	
14	6.4 "	$<1.0 \times 10^2$	
15	5.6×10^6	Discontinued	
20	3.2 "		
25	8.1×10^7		
26	1.8×10^8		
27	8.4×10^7		

dicated a poor state of tissue cultures. Similar findings were obtained with 3 other definitive but less extensive transfer series. In the experiment here described, contamination occurred at 27th transfer, forcing discontinuation of the series. The results justify the assumption that intracellular growth involving transfer through guinea pig monocytes can be maintained practically indefinitely.

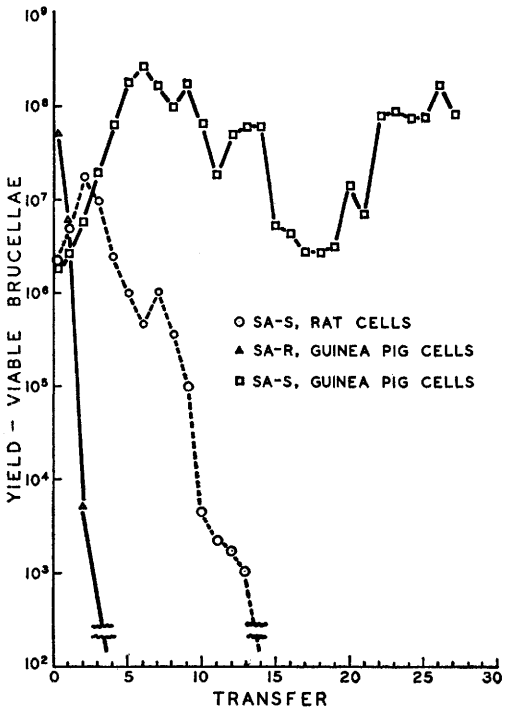

FIG. 1.

Differences between growth of Br. abortus *in guinea pig- and rat-cell cultures.* Fig. 1 and Table I show that in our system, at least, rat mononuclear cell cultures do not support continued growth of SA-S in a manner comparable to that observed during continuous transfer in guinea pig cells. A gradual decline of viable count with complete loss of recoverable brucellae by transfer 14 occurred. Similar trends were observed in 2 other experimental series. It should be mentioned that rat cells kept under our culture conditions generally appeared more rounded than healthy guinea pig monocytes. This is usually indicative of cells in poor condition. Based upon our experience with guinea pig monocytes, however, these changes were not of sufficient magnitude to account for the striking decrease in brucellae observed in rat-cell transfers.

Lack of growth of rough variants. Prior investigations(3) had demonstrated inability of avirulent rough *Br. abortus* organisms to multiply in guinea pig mononuclear cells. This was again confirmed by observations that SA-R bacteria are rapidly eliminated from guinea pig- and rat-cell cultures (Fig. 1) and Table I. This evidence reemphasizes the utility of the present method in demonstrating strain differences in ability to survive and multiply in intracellular environment.

Cultural characteristics of isolates. To check for possible occurrence of changes in characteristics following continuous multiplication in an intracellular environment, tests were made on stability of a number of easily detectable characteristics of bacteria harvested after various periods of continuous transfer through monocytes. The characteristics tested included (1) possible changes in colonial morphology, as detected by plating of aliquots of the harvest fluid directly onto tryptose agar with and without triphenyltetrazolium or onto 2-1 agar, (2) sensitivity to thionin and basic fuchsin, (3) capability of growing in air and CO_2 (SA-S is an aerobic mutant), and (4) agglutinability by specific antiserum. No detectable alterations in these

characteristics were found. However, the potential for population changes under conditions employed was indicated by the fact that a few rough (R) colonies present in the initial predominantly smooth (S) inocula disappeared after one or 2 transfers, suggesting that the technic of continuous transfer is capable of selecting out S types from a mixed population. Other changes, such as alterations in virulence and immunogenic characteristics may have occurred during these transfers, but their detection requires more extensive tests, now under way.

Discussion. Brucellae are capable of surviving and multiplying within the cytoplasm of certain host cells(5,6). Therefore, continuous growth in a corresponding micro-environment *in vitro* may provide means for maintaining the organism in a physiologic and genetic state approximating that found in naturally infected animals. As demonstrated with some bacteria(7), antigenic differences between genetically apparently identical organisms grown under different environmental conditions can occur and, therefore, differences between slant-grown and intracellularly grown brucella could be expected. Although detection of such possible differences will require further work, the present study has led to development of a method for investigating these and related factors of importance in pathogenesis.

Results of comparison of growth within guinea pig mononuclear leukocytes and rat mononuclear leukocytes correlate with what is known about the *in vivo* fate of *Br. abortus* in these animals. Guinea pigs are susceptible to small numbers of S. brucellae, and the organisms can be recovered from spleen or lymph nodes long after initiation of infection. In contrast, rats seem to possess a higher degree of natural resistance(8,9). Correlation between such information and our results suggests that resistance at the cellular level may be of considerable importance in determining such differences in host susceptibility. The slow loss of brucellae in the rat mononuclear system may indicate that subtle interference with intracellular multiplication, rather than strong bactericidal factors, is involved. Thus, in the rat, innate resistance to brucellosis may depend, at least in part, upon innate activity of the phagocytic cells themselves. Later in the disease, immune factors may become more important. On the other hand, in the guinea pig, whose exposure to small numbers of virulent bacteria results in a progressive but, eventually, limited disease, resistance may depend upon *subsequent* modification of state of resistance of the phagocytic cell. It has been shown that virulent brucellae are capable of multiplying within monocytes from normal guinea pigs, but multiplication is inhibited within monocytes from infected or immune animals(1). Thus, in a susceptible host such as the guinea pig, the brucellae initially depend on favorable intracellular environment for nutrients, but subsequently encounter an interference with multiplication. This interpretation of present data presumes that no significant modifications of the mononuclear cells have occurred during their *in vitro* maintenance which would alter resistance factors so that extrapolation to *in vivo* conditions would be misleading.

The basis for the observed interference with bacterial multiplication in rat mononuclear cells remains a matter for speculation. It may involve effective yet weak antibacterial intracellular factors, it may also be due to a lack of an essential nutrient for bacterial multiplication, or it may involve some other metabolic factor. In connection with the latter possibility, it is interesting to note that Wallace and his associates(10) have demonstrated a profound effect of hydrocortisone on intracellular multiplication of *Mycobacterium lepraemurium*, an effect that is presumably due to modification of metabolism of the infected cell and that correlates well with the known effects of this and related hormones in certain diseases.

In apparent contrast to our observations, Holland and Pickett(4) have reported growth of brucellae in rat mononuclear cells. However, we have noted similar evidence of multiplication in non-continuous rat-cell cultures. This may be an expression of a limited amount of residual intracellular growth following growth on lifeless media. It should be remembered that only a few cell divisions are

required to pack a mononuclear cell with bacteria.

Summary. Continuous intracellular growth of virulent *Brucella abortus* organisms, within mononuclear cells of guinea pigs, by periodic transfer from tissue culture to tissue culture has been accomplished. Comparable continuous growth could not be accomplished using rat-monocyte cultures. Continuous transfers were unsuccessful in the case of rough avirulent organisms in guinea pig- and rat-monocyte cultures. The bearing of these observations upon host and species resistance has been discussed.

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Virologic Use of Monkey Kidney Cells Preserved by Freezing.*† (24963)

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Because of their susceptibility to a broad spectrum of viruses affecting man, as well as their necessity in production of vaccines for human use, cultured Rhesus monkey kidney cells continue to serve as an essential virologic tool. There are, however, many well-known difficulties involved in their use, such as availability and viral infections acquired *in vivo*(1), which might be circumvented if such cells could be preserved until they are needed or tested. Ability to preserve mammalian cell strains(2-5) and certain animal tumors(5) by freezing suggested that intact cells enzymatically separated from their host tissues might be successfully treated in a similar fashion. This communication describes a procedure of freezing and storage as efficient and practical means of preserving cells of freshly trypsinized Rhesus monkey kidneys as well as cells of primary cultures. Also described are their sensitivities to a variety of viruses as determined by cytologic response and assays by tube titrations or plaque counts.

Procedures. Preparation of cell suspensions. Two types of cell suspensions were frozen: 1) freshly trypsinized Rhesus monkey kidney prepared in the laboratories of Parke, Davis & Co., by methods routinely employed for production of virus vaccines, and 2) versene-treated monolayers of primary cultures of monkey kidney that had been grown in Eagle's basal medium containing 5% calf serum. In both cases the cells were resuspended in freeze medium which consisted of Eagle's basal medium 80%, calf serum 15%, and glycerol 5%. Concentrations of living cells were determined by adding 0.1 ml of 0.1% solution of neutral red to 1 ml of cell suspension, and the mixture incubated 30 minutes at 37°C. Up to 4 ml aliquots containing 0.8 to 2 x 10^6 living cells/ml were transferred to 5 ml vials and sealed with apron rubber stoppers. Sufficient concentrations of cells were frozen to

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