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Interactions Between Mononuclear Phagocytes and *Brucella abortus* Strains of Different Virulence.* (23752)

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In prior studies on the multiplication of *Brucella abortus* in mononuclear phagocytes (monocytes) of guinea pigs, maintained *in vitro,* it was noted that after different periods of incubation some monocytes always tended to contain considerably more bacteria than others(1). It was suspected that such heterogeneity may have been due in part to a genetic heterogeneity among members of the bacterial population in regard to characteristics influencing ingestion by phagocytes and intracellular multiplication. It is well known that virulent smooth (S) and relatively avirulent rough (R) types are phagocytosed at different rates by polymorphs. In addition, it has been shown that nonsmooth *B. abortus* mutants fail to multiply within chick embryo fibroblasts capable of supporting multiplication of smooth type cells(2). Furthermore, when the culture used in the prior quantitative studies(1) was examined 2 years later, appropriate tests(3) revealed that it contained approximately 20% R types in addition to S cells. Therefore, additional studies were initiated to determine the extent to which differences in antigenic and virulence characteristics of the bacteria may influence their interactions with monocytes from normal or immune guinea pigs.

Methods. Tissue culture technics followed essentially those detailed previously(1). Monocytes were harvested from guinea pigs 3-4 days following intraperitoneal injection of 2 ml sterile mineral oil. They were washed, counted and suspended in Hanks solution containing 30% homologous serum to yield a concentration of approximately 1 x 10^6 cells/ml. Bacteria harvested from 18-hour-old tryptose agar cultures were added to yield concentrations approximating either 2 x 10^8/ml (*high* inoculum) or 2 x 10^7/ml (*low* inoculum). 1.5 ml of such suspensions were placed into rubber-stoppered Porter flasks containing flying cover slips, and incubated at 37°C. Two hours later the liquid was replaced by serum-Hanks' containing 10 μg of streptomycin per ml. It was established that this concentration of antibiotic will kill all except $<0.001\%$ of the extra-cellular bacteria

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TABLE I. Data Obtained from One Maintenance Culture of Normal Guinea Pig Monocytes Exposed to a Mixed S + R Population of *B. abortus*, 6232.

Bacterial counts	% S
Initial (F*): 2×10^8	55
From supernate of 72-hr-old culture (L*) approx. 2000	64
From supernate of harvested washed mono's (L), approx. 300	79
Within mono's, final (F): 5.5×10^5	90
Microscopic observations	
Infected mono's: 98%	
Mono's with >10 bacteria: 65%	
" " >25 " 41%	

* F = per flask; L = per loopful.

within 2-6 hours but, apparently, will not interfere with their intracellular multiplication. After a given period of incubation (usually 24, 48, or 72 hours after introduction of the streptomycin-serum-Hanks' medium), cover slips were removed from the Porter flask cultures and stained; the remaining monocytes were harvested and washed as previously reported(1). Samples from the supernatant (*Su*) of washed, centrifuged monocytes were plated on 2-1 agar(4) for determination of colonial morphology of the bacterial progeny, and viable counts were made by plating appropriate dilutions on tryptose agar. The final bacterial count resulted from suspending and diluting the washed, sedimented monocytes in *plain* Hanks' which apparently sufficed to release the intracellular bacteria from the leukocytes. Table I and Fig. 1 indicate the kind of data collected for each of the 100 monocyte cultures on which the present report is based. The bacterial strains employed in this study included either S or R types isolated from an air-growing culture of *B. abortus*, SA, and from a CO_2-requiring culture of *B. abortus*, 6232. The SA culture was originally isolated from a human case in Puerto Rico, and *B. abortus* 6232 originated from an infected cow. In addition, a smooth *B. abortus*, strain 19, culture was used in a few experiments.

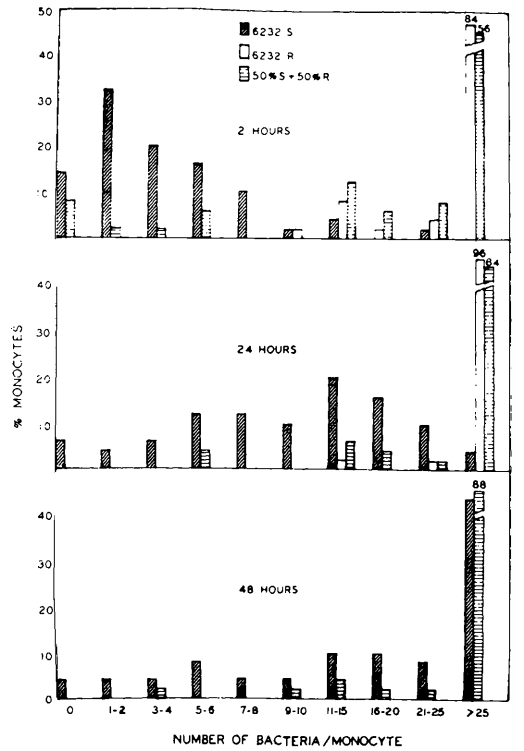

FIG. 1. Distribution of number of *B. abortus* per monocyte after various periods of incubation.

TABLE II. Final Bacterial Count ($\times 10^3$) per Flask after 48 Hours of Incubation.

Bacterial inoculum	Exp. #5	Exp. #7
SA-S	352.1	90.0
-R	7.3	3.5
6232-S	4.8	not done
-R	.6	"
-S in CO_2	20.8	"
-R "	9.0	"

Results. Differences between S and R strains. A large series of bacterium-monocyte cultures, initiated either with SA-S, SA-R, 6232-S, 6232-R, or mixtures of corresponding S + R bacteria, indicated that virulent S cells multiplied in monocytes from normal guinea pigs, whereas the relatively avirulent R type cells either failed to do so or multiplied only to a limited extent. Such differences in the capacity for intracellular growth were detectable by comparison of the final viable counts of bacteria (Table II), by the pattern of distribution of number of bacteria per monocyte after various periods of incubation (Fig. 1), by comparing (Table III) the relative ratio of bacteria harvested from disintegrated monocytes (final count) to number of extracellular bacteria recoverable from the supernatant (*Su*, see methods), and by comparison of the ratio of the final count

TABLE III. Ratio of Number of Bacteria Harvested from Washed Monocytes to Number Recoverable from Supernatant.
(Age of cultures: 72 hr.)

Bacterial inoculum		Ratio
SA-S	"high"	51.4
-R	"	6.1
6232-S	"	5.9
-R	"	2.3
Strain 19-S	"	.8
SA-S	"low"	10.9
-R	"	3.8
6232-S	"	5.4
-R	"	2.7

from 48-hour-old cultures with the final count from corresponding 2-hour-old cultures (Table IV). In addition, the increase in % S in bacterial populations harvested from cultures originally inoculated with a mixture of S + R (Table I) reflected the differential multiplication of virulent and relatively avirulent types.

Observations on the distribution of number of bacteria per monocyte showed significant differences between S- and R-exposed cultures, particularly during the early stages of bacterium-monocyte interactions. As illustrated in the "2-hour" example of Fig. 1, the majority of monocytes exposed to an excess of S bacteria contained 1-6 bacteria shortly after initiation of the Porter flask cultures; in contrast, monocytes exposed to R bacteria were virtually loaded with bacteria at this stage. Such differences can be attributed to a number of causes, including the tendency of R type bacteria to clump and to be ingested with greater ease than S bacteria. In addition, this difference may be indicative of some type of change occurring in monocytes that have ingested one S bacterium, which may interfere with the continued ingestion of additional S bacteria during the first 2 hours. However, regardless of the actual causes for the early differences in number of S *vs.* R bacteria per monocyte, the existence of such pronounced differences seems to permit their utilization for the simple and rapid detection of the extent of homogeneity or heterogeneity existing in the bacterial population that was employed as inoculum (see 2-hour data in Fig. 1, including the bimodal distribution in the case of a mixed S + R inoculum). The described differences tend to disappear with increasing time of incubation (Fig. 1) due to a gradual shift to higher numbers of bacteria per monocyte in cultures with S bacteria; this shift undoubtedly is a reflection of the intracellular multiplication occurring in such S cultures.

One additional phenomenon is reflected in Fig. 1 by the absence of any 48-hour counts for monocyte cultures initially exposed to R bacteria. The lack of such determinations is due to the almost total absence of nondisrupted monocytes, which reflects the rapid rate of destruction of monocytes following ingestion of large numbers of R bacteria. Many of the S-containing monocytes also eventually disintegrated, but this did not occur until later periods when large numbers of bacteria per monocyte had been attained due to intracellular multiplication.

Differences between S strains. In all experiments, strain SA-S displayed the most rapid rate of intracellular multiplication during the first 48 hours. Bacteria of strain 6232 S (CO_2-requiring) multiplied at a considerably slower rate in rubber-stoppered cultures, and this tardy rate was not brought up to that of SA-S even when cotton-plugged cultures were incubated in CO_2 (Table II). However, after 72 hours of incubation (see "Normal" data in Table V), the CO_2-requiring bacteria appeared to catch up with the non-CO_2 requirers as far as final counts obtained from cultures showing similar survival of monocytes are concerned. Microscopic observations of monocytes from representative cultures have revealed no significant differences in rate and extent of ingestion of 6232-S or SA-S bacteria. Therefore, such observations would indicate that the observed strain dif-

TABLE IV. Ratio of Final Bacterial Count from 48-Hour-Old Cultures to Final Count from 2-Hour-Old Cultures.

Bacterial inoculum		Ratio
SA-S	"high"	4.6
-R	"	.05
-S	"low"	8.3
-R	"	.27

TABLE V. Final Bacterial Count ($\times 10^3$) per Flask in 72-Hour-Old Cultures Initiated with Immune or Normal Monocytes.

Bacterial inoculum	Monocytes and serum	Count
SA-S	Normal	317.5
"	Immune	13.0
6232-S	Normal	375.0
"	Immune	1.5
SA-R	Normal	15.0
"	Immune	.8
6232-R	Normal	30.0
"	Immune	1.3

ferences are due primarily to differences in the duration of the lag period prior to initiation of intracellular multiplication. It remains to be determined to what extent these differences may be associated with strain differences in virulence. S (perhaps more properly labelled I_1) bacteria from the immunogenic, relatively avirulent *B. abortus*, strain 19, displayed an exceptional behavior. They failed to multiply intracellularly and appeared to disintegrate rapidly in normal guinea pig monocytes. This is in contrast to the microscopically visible retention of morphological integrity of other S and R bacteria studied. However, there is a resemblance between the morphological and physiological interactions of strain 19 bacteria with *normal* monocytes, and the interactions observable when bacteria from virulent strains are ingested by monocytes from *immune* guinea pigs.

Interactions with immune monocytes. A limited number of cultures containing immune serum were initiated with monocytes from immune (previously infected) guinea pigs and S or R bacteria. In all instances ingestion of bacteria was fast, and was followed by very rapid bacterial and cellular destruction. Little or no intracellular multiplication of S bacteria occurred (Table V). There was some indication that, in contrast to the effects noted with normal monocytes, S bacteria may destroy immune monocytes more rapidly than R bacteria.

Miscellaneous observations. With an increase in the size of the bacterial inoculum, increases in the extent of ingestion, rate of monocyte destruction and final counts after a given period of time were observed. In addition, it was noted that quite frequently a large proportion of bacteria harvested from monocyte cultures gave rise to tiny colonies; this phenomenon probably is indicative of some temporary modification (injury) suffered by the clonal parent since subcultures from these tiny colonies yielded colonies of normal size.

Discussion. The foregoing observations indicate that S and R types of *B. abortus* differ not only in their capacity for intramonocytic multiplication, as they do in the case of chick fibroblasts(2), but that they also differ in regard to the rate with which they are ingested and with which they subsequently destroy monocytes. The low initial extent of multiple ingestion of S bacteria by individual monocytes may be due either to a specific interference with continuous ingestion or merely to an injury to the phagocyte caused by prior contact with an S type cell. In contrast, the less virulent R type bacterium may be less injurious and thus leave the phagocyte with the continued capacity for ingestion. "Superinfection" experiments with labelled S and R strains will be required in order to elucidate this point. The more rapid destruction of normal monocytes by R than by S bacteria may be a reflection of the need for attainment of a minimum number of bacteria per normal monocyte prior to initiation of monocyte destruction. The required intracellular number of bacteria would be attained rapidly by continued early ingestion in the case of R types, but more slowly by intracellular multiplication in the case of S types. An even more rapid destruction of monocytes that was observed to occur following exposure to S + R mixtures, possibly due to the combined effects of intracellular multiplication (S) and initial multiple ingestion (R).

The observed alterations of bacterium-monocyte interactions *in vitro* due to differences in bacterial population heterogeneity, strains, and inoculum sizes, stress the need for a certain degree of standardization of procedures and materials as a prerequisite for comparing results obtained in different laboratories.

It is noteworthy that even following the use of relatively homogeneous bacterial in-

ocula, there remained a certain amount of bimodality in the distribution of number of bacteria per monocyte in cultures older than 24 hours. This would suggest that in the present and past(1) studies some unrecognized heterogeneity persisted either in the smooth bacterial populations (in regard to properties influencing interactions with mammalian cells) or in the phagocyte population. Preliminary experiments have indicated that differences in the age of individual monocytes at time of initiation of the cultures may account at least in part for such heterogeneity.

Finally, the observations on monocyte destruction *in vitro* suggest certain future studies in regard to the extent to which similar phenomena *in vivo* might modify the course of disease caused by facultative intracellular bacteria such as *Brucella*. Since it is likely that for these organisms residence and multiplication within phagocytes represents an advantage for the pathogen (*e.g.*, protection from bactericidal blood factors and certain antibiotics), induced monocyte destruction by avirulent R type cells, possibly even by appropriate inert particles or other methods, may contribute to the amelioration of the disease. It is possible that such an approach already may have been employed unwittingly in the case of combined tuberculin and antibiotic treatment(5).

Summary. Studies on the *in vitro* interactions between S and R strains of *Brucella abortus* and monocytes from normal or immune guinea pigs have indicated 1) intracellular multiplication of S bacteria and a lack of, or insignificant extent of, multiplication of R bacteria in normal monocytes, 2) differences in the rate of ingestion of S and R bacteria, 3) differences in the rate of destruction of monocytes by S and R types, 4) differences in the rate of intracellular multiplication among S strains, 5) a significant modification of these events in immune monocytes, and 6) an apparently unique behavior of immunogenic, relatively avirulent strain 19-S bacteria in normal monocytes. The relation of these observations to some problems of host-parasite interactions is discussed. It also has been shown how these observations may provide the basis for a new technic of rapid assessment of the homogeneity or heterogeneity of bacterial populations in regard to properties associated with virulence.

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Leukemia. VIII. Leukemogenic Effect of Brain Filtrates After Serial Passage Through Mice.* (23753)

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Because of the purported ability of cell-free brain extracts to induce leukemia in mice (1-4), experiments were undertaken to test further the validity of the claim that the filtrates were cell-free. The experiments were so devised that they would give evidence also of the presence of a self-perpetuating agent, and would allow for the comparison of cell-free brain filtrates with tumor homogenates as leukemogenic agents.

The demonstration by Furth(5) that a single cell injected intravenously may produce a lymphoblastoma in mice and the reports of Stasney, Cantarow and Paschkis(6,7)

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