SOME OBSERVATIONS ON THE FILTRABILITY OF BACTERIUM TULARENSE

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Throughout our morphologic study of Bacterium tularense in liquid medium cultures we noted constantly the presence of very minute forms (Hesselbrock and Foshay, 1945). These appeared to grade downward in size from the most prevalent coccoid or ring forms of about 0.5 μ in diameter to bodies so small that neither size nor shape was discernible. Such particles were never seen in uninoculated media, either before incubation or for 96 hours thereafter. Some particles were discrete and freely suspended; others were attached by delicate short filaments to larger morphologic units. Many additional ones were seen lying in, and apparently arising within, delicate filaments of variable length. The number of discrete particles was never large at any time in any of the many cultures that were examined. Single particles in suspension, as well as those filamentously attached or interconnected, stained a brick-red color when supravirtually stained with 2 per cent aqueous malachite green, whereas all large morphologic units stained green. None of six other common pathogenic bacteria, cultivable in the same medium, produced particles of this size. Prolonged intermittent observations of cultures, although inadequate to establish the fact, suggested strongly that these particles were capable of reproducing all other forms of this pleomorphic organism and, apparently by means of larger units, of reproducing themselves. Our provisional interpretation was that they represented true morphologic units of Bacterium tularense, possibly to some degree analogous in size and function to the minute elementary bodies formed by organisms of the pleuropneumonia group, appropriately called minimal reproductive units, M.R.U. (Sabin, 1941).

It has long been known that virulent strains of the organism sometimes, in about one half the recorded trials, pass Berkefeld, Chamberland, and Seitz filters. We cannot find reports on the morphology of the units that passed the filters, but our observations suggested that the provisionally designated M.R.U. might have accounted for the infectivity of the filtrates. To test our views we used gradocol membranes for ultrafiltration of virulent cultures.

MATERIALS AND METHODS

Two equally virulent strains were used. Throughout the 21 months during which filtrations were spaced, frequent titrations in white mice gave LD₆₀ titers between 9.3 and 9.5. Infrequent tests in rabbits, using suspensions of T-860 × 10⁻⁶ dilution (initial turbidity equivalent to that of the standard U.S.P.H.S.

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² Captain, S. C., A. U. S.
agglutination test suspension), killed all animals within 5 days. Two filtrations were performed after cultivation for 48 and 72 hours, respectively, in a gelatin-hydrolyzate glycerol cystine liquid medium. The third filtration was performed with a pooled saline suspension of growths from 4-, 5-, and 6-day cultures on blood glucose glycerol cystine agar. All initial turbid suspensions were cleared by centrifugation before passing the supernatants through the preliminary screening membranes.

Original Elford gradocol membranes were used with low positive nitrogen pressure. Immediately prior to filtration all membranes were satisfied by passing about 12 ml of beef heart infusion broth of pH 7.7. The entire culture supernatant was then filtered through a coarse membrane. The first liquid culture and the solid media culture supernatants were screened through membranes of 900 m\(\mu\) A.P.D. After removing samples the filtrates were divided, and equal portions were simultaneously filtered through membranes of 600, 500, 400, and 300 m\(\mu\) A.P.D. The second liquid culture supernatant was screened through a 770 m\(\mu\) A.P.D. membrane, then treated as were the others. The major portion of each filtrate was obtained by gravity filtration. Pressures of 2 lb/sq in were applied until filtration ceased; then terminal pressures of 10 lb/sq in were applied to ensure completion. Inspection of all membranes after filtration revealed no defects.

Samples of each filtrate were treated as follows: Large loopfuls were examined by dark-field illumination; from 0.20 to 1.0 ml amounts were inoculated into duplicate series of gelatin hydrolyzate medium; large loopfuls were transferred to slants of blood glucose glycerol cystine agar in duplicate; either 0.5 or 1.0 ml was inoculated intraperitoneally into groups of 5 or 10 mice each; and from 0.1 to 1.0 ml was cultured on 20 per cent serum agar and in 20 and 30 per cent serum broths. Two preliminary screening filtrates, one each from 900 m\(\mu\) and 770 m\(\mu\) A.P.D. membranes, were titrated for numbers of infective units by intraperitoneal inoculation into mice of 0.5 ml quantities of ascending twofold dilutions.

Necropsies were performed on all mice that died. Heart blood cultures were made and a portion of each liver was removed for histologic study. The constancy with which this disease produces characteristic lesions in the mouse liver, hepatic cell pseudocysts filled with bacteria, and typical foci of necrosis makes this organ an extremely reliable one for detection and verification of tularemic infection.

**EXPERIMENTAL OBSERVATIONS**

*Solid medium cultures.* The supernatant of pooled solid medium cultures gave evidence of passing only the 900 m\(\mu\) A.P.D. membrane. All mice inoculated with this filtrate died on the third day; they yielded heart blood cultures pure for *Bacterium tularense*, and liver sections that showed typical tularemic lesions. Cultures were readily obtained from solid and liquid media that contained cystine. All cystine-free media remained sterile despite careful search for pleuropneumonialike organisms. The filtrate was unexpectedly rich in infective units, killing all mice in our highest dilution so that an end point was not secured.
Filtrates from 600, 500, and 400 m\(\mu\) A.P.D. membranes remained sterile after cultivation in all media, and failed to kill any mouse of lots of 10 each, inoculated intraperitoneally in 1.0 ml amounts.

**Liquid medium cultures.** The culture supernatant that was screened through a 900 m\(\mu\) A.P.D. membrane yielded infective filtrates from the 900 and 600 m\(\mu\) A.P.D. membranes but not from membranes of lesser A.P.D. Discrete particles were observed by dark-field illumination only in the two filtrates that later proved to be infective. With the minimal inocula stated above, all filtrate cultures remained sterile. The 900 m\(\mu\) A.P.D. membrane filtrate was not titrated for infectivity. All of 10 mice inoculated with 0.5 ml each of this filtrate died; they yielded heart blood cultures positive for *Bacterium tularense*, and liver sections with typical tularemic lesions. Of 10 mice each inoculated with 0.5 ml of the 600 m\(\mu\) A.P.D. membrane filtrate, 4 died with evidences of tularemia. Two of these gave heart blood cultures pure for *Bacterium tularense*, and liver sections that were typical for this infection. The other two gave heart blood cultures positive for *Salmonella*, and liver sections characteristic for “mouse typhoid” but with a few tularemic lesions also. Of the remaining 6 mice, those that died were shown by cultures and sections to have died of *Salmonella* infection. Neither cultural nor histologic evidence of tularemia was found for any dead mouse inoculated with a filtrate from a membrane with an A.P.D. smaller than 600 m\(\mu\).

The culture supernatant that was screened through a 770 m\(\mu\) A.P.D. membrane yielded a cultivable and infective filtrate only from the 770 m\(\mu\) A.P.D. membrane. Direct cultures grew *Bacterium tularense* but no other organism. All of 10 mice each inoculated with 0.5 ml of this filtrate died; they yielded heart blood cultures positive for *Bacterium tularense* only, and liver sections with characteristic tularemic lesions. Titration of this filtrate for infectivity, in serial twofold dilutions in lots of 5 mice each, revealed that the 1:4 dilution killed exactly 50 per cent of the mice. Except for one death by accidental trauma, all mice, 10 in each lot, survived inoculation with filtrates from membranes of 600, 500, 400, and 300 m\(\mu\) A.P.D.

**Morphology of cultures derived from filtrable forms.** The two cultures derived from heart bloods of mice inoculated with filtrate from a membrane of 600 m\(\mu\) A.P.D. were subjected to morphologic study by methods previously described. Each culture developed all morphologic units previously found to be constant and characteristic for the species—coccoid and ring forms, flattened and rounded bacillary forms, globules, filaments, and minimal reproductive units both solitary and filamentously attached and interconnected (Hesselbrock and Foshay, 1945).

**Pathologic changes in livers of white mice.** When mice are fatally infected by the ocular or cutaneous routes with whole cultures or with infected tissues, or by subcutaneous or intraperitoneal injections of moderate to large inocula of culture suspensions, the characteristic pathologic changes in the livers are (1) numerous foci of necrosis with extensive karyorrhexis and (2) a small to moderate number of pseudocysts, swollen hepatic cells completely filled with *Bacterium tularense*.

All mice fatally infected by membrane filtrates showed a reversed ratio of
these hepatic features, numerous large pseudocysts and few to moderate numbers of necrotic foci. This remarkable alteration in liver lesions was not ascribable to the nature or size of the infecting morphologic unit but probably to the number since identical changes were consistently produced by inocula of suspensions of whole cultures in serial decimal dilutions from $10^{-4}$ upwards.

DISCUSSION

Our original aim to determine critically the dimensional limits of the M.R.U. remains unfulfilled and is reluctantly postponed due to current conditions of work. Nevertheless it was demonstrated that morphologic units that passed a 600 $\mu\text{m}$ membrane but not a 500 $\mu\text{m}$ membrane, and hence were in the range of 300 to 350 $\mu\text{m}$ in diameter, were infectious, and that their development resulted in the complete series of morphologic units now known to be characteristic for Bacterium tularense. At no time, from either cultural or animal sources, were we able to demonstrate the presence of any associated or concomitant microorganism other than the easily identified “contaminants” from mice.

Since 0.5 ml of a 1:4 dilution of a 770 $\mu\text{m}$ membrane filtrate was the LD$_{50}$ dose, it is apparent that our cultural conditions did not yield filtrates rich in M.R.U. A true estimate of the size of the smallest units will require filtrates containing many more M.R.U. Since a large proportion of M.R.U. observed at any time during 8 days of cultivation in gelatin hydrolyzate medium was always found to be within filaments, or filamentously attached to larger morphologic units, the cultural needs are those that will yield a high proportion of discrete particles of the smallest size. Knowledge of the rate of development of M.R.U. to larger units is also desirable.

SUMMARY

The filtrable form of Bacterium tularense in gradocol membrane filtrates of gelatin hydrolyzate medium cultures of highly virulent strains produced tularemia in the highly susceptible mouse and was recoverable in the form of larger morphologic units by heart blood cultures. These filtrable forms were approximately 300 to 350 $\mu\text{m}$ in diameter. We are reasonably sure that minimal reproductive units of smaller size exist, and that accurate measurement will require better cultural conditions than those hitherto obtained. Morphologic studies of cultures obtained from mice inoculated with filtrates demonstrated that the filtrable units were capable of development into all other morphologic units known for this organism.

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REFERENCES
