STUDIES ON THE VARIATION OF BACTERIUM TULARENSE

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Although the virulence of *Bacterium tularense* may be maintained for prolonged periods of cultivation on artificial media, a considerable reduction in virulence has been frequently observed. As early as 1922 Francis reported the loss of virulence of certain strains of *B. tularense* following serial transfer on serum glucose agar. Foshay (1932) described the loss of virulence of two strains isolated from fatal human cases following prolonged cultivation on coagulated egg yolk medium. Ransmeier (1943) reported a reduction of virulence after serial transfer of an originally highly virulent strain on glucose cystine blood agar. It is also well known that repeated passage of cultures through a given animal species may result in a marked increase in virulence for the particular host (Philip and Davis, 1935). Thus, changes in virulence have been amply demonstrated; however, it has not been possible to correlate these changes with any other easily detectable characteristics. All attempts to correlate cellular morphology with virulence (Hesselbrock and Foshay, 1945; Eigelsbach, Chambers, and Coriell, 1946) have met with failure.

Because various bacterial species display a high degree of correlation between colonial morphology, pathogenicity, and immunogenic properties (Braun, 1947), an attempt was made to determine whether such correlations may also exist for *B. tularense*. As far as the authors are aware, studies on the colonial morphology of *B. tularense* have not been reported previously in the literature.

MATERIALS AND METHODS

The majority of experiments were initiated with either a highly virulent culture, Schu, originally isolated by Foshay in 1941 from a human ulcer, or with an avirulent culture, 38, which was originally isolated by Francis in 1920 from a human lymph node and subsequently became totally avirulent (Hesselbrock and Foshay, 1945). These cultures, as well as variant types subsequently isolated, were maintained on glucose cysteine blood agar (GCBA) at 5 to 10 C following a 24-hour growth period at 37 C.

The solid transparent medium used for viable count and colony type observations consisted of 2 per cent Difco peptone, 1 per cent NaCl, 0.1 per cent glucose, 0.1 per cent cysteine hydrochloride, and 2 per cent agar with a final reaction of pH 6.8 (Snyder *et al.*, 1946). The basal liquid medium used was identical in composition except for the absence of agar. Viable counts were made by plating serial dilutions on GCBA; total growth was determined by packed cell volume in modified hematocrit tubes after centrifugation at 4,000 rpm for 1 hour.

Colony types were inspected under a dissecting microscope with the help of obliquely transmitted light, achieved by placing the mirror, concave side up,

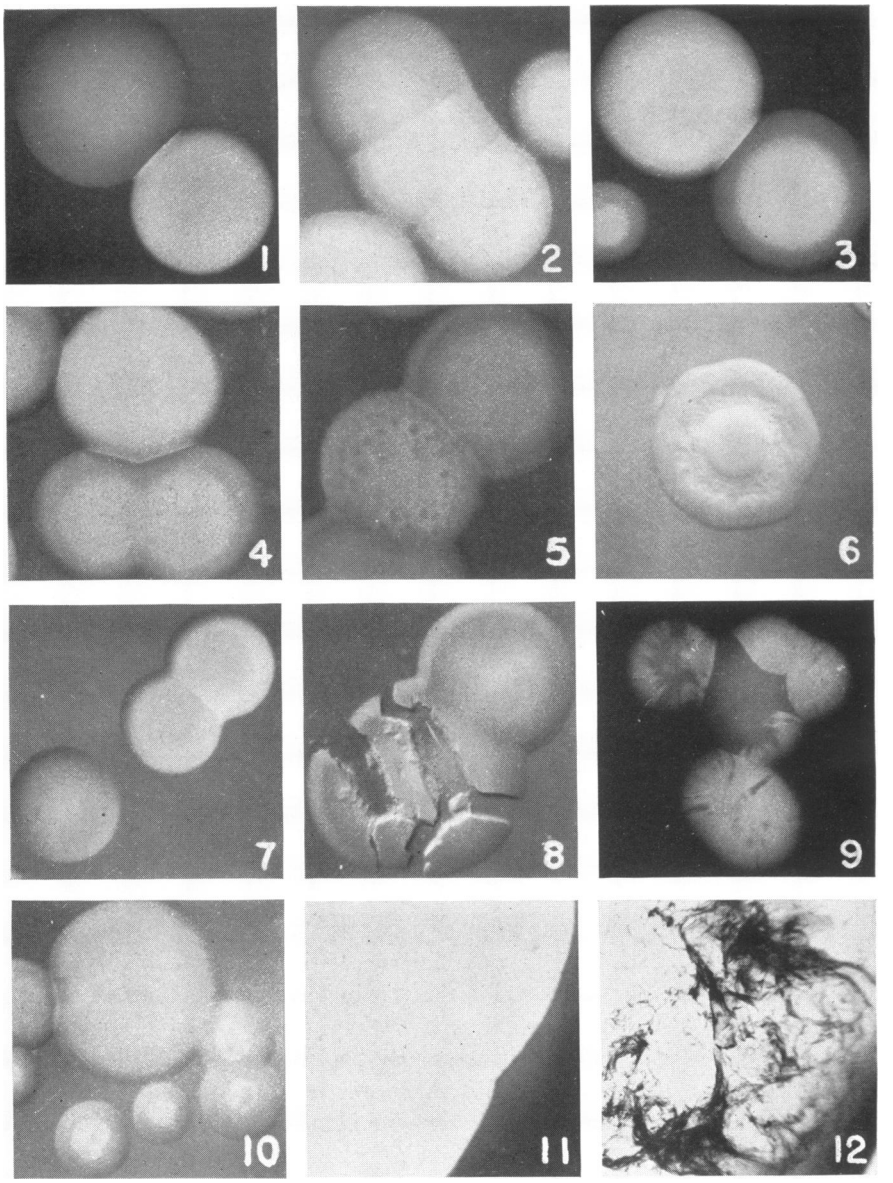

Figure 1. Colony type variants of *Bacterium tularense* and their acriflavine reactions. ×100. In the following descriptions of colony type variants, the typical acriflavine reaction is expressed as an exponent whenever it cannot be correlated with colony appearance.

1. Smooth, blue Schu S_4 colony and smooth, dense, buff Schu S_2 colony. Colonies are round, raised, and convex.

2. Three smooth colony types of Schu: (1) less dense, blue Schu S_4; (2) more dense, buff Schu S_1; (3) dense, buff Schu S_2. Type Schu S_4 is watery in consistency, whereas types Schu S_1 and S_2 are buttery.

approximately 3 inches in front of the substage. Illumination was provided by a microscope lamp that was located at a level higher than that of the mirror (Henry, 1933). The distance between the mirror and the lamp was approximately 7 inches. Samples of cultures were streaked on the surface of 20 ml of solid medium, contained in a flat-bottom petri plate, in such a fashion that dense areas of growth as well as isolated colonies were obtained. These plates were examined after 48 to 72 hours of incubation at 37 C. Additional special methods will be cited in the description of results below.

RESULTS

Colony types. With the aid of the oblique lighting arrangement and the transparent solid medium, it was possible to recognize a small number of distinctly different colony types in supposedly pure stock cultures of strains Schu and 38. Subsequent examinations of many other stock strains confirmed their general heterogeneity in regard to colony types. Differences in color and opacity, usually not observable by ordinary illumination from a mirror directly under the objective of the microscope, were easily discernible by the modified lighting method. A considerable variety of smooth (S), convex colony types could be noted. They differed in density and color but usually showed a similar buttery consistency. Nonsmooth (NS) colony types were usually distinguished by granular appearance and brittleness when touched with a needle. Examples of colony types are presented in figure 1. Whenever a colony type variant was observed, the colony or a section of a colony was picked and usually gave rise to stable homogeneous subcultures. A few unstable types were observed (see figure 1, no. 9) in the process of such isolations of variant cultures.

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3. Schu S_3 is shown at the lower right immediately adjacent to a Schu S_2 colony. The center of Schu S_3, which is surrounded by a blue peripheral zone, is less dense than Schu S_1 or S_2 but more dense than Schu S_4.

4. Gray, viscous Schu NS_2 colonies, adjacent to a single Schu S_2 colony. Note the granularity of the NS_2 colony type, which is of greatly reduced virulence and when suspended in saline agglutinates with acriflavine as shown in figure 1, no. 12.

5. Daughter colonies arising from a Schu S_2 colony after prolonged incubation.

6. Single colony of Schu NS^a. This rough-appearing colony type possesses a central plateau surrounded by an irregular pitted area that gradually blends with a smooth peripheral zone. This colony type does not agglutinate with acriflavine and is of high virulence for mice.

7. Three variant colony types of strain 38. At the lower left, gray $38S_1^{ns}$; in the middle, $38S_2^{ns}$ dense, irridescent $38S_2^{ns}$; and at the upper right, dense, buff $38NS_1$.

8. Colonies of $38NS_2$. Colonies of this variant are dense, firm, dry, dull, flat, and light gray. They are extremely brittle and, when touched with a wire, break into several pieces with sharp, irregular edges as illustrated in the lower portion of the photograph.

9. Sectored colonies arising from certain isolates of strain 38 and typical of unstable variants.

10. A blue 38 S_1 colony and several gray dwarf $38NS_d$ colonies.

11. A negative acriflavine slide-reaction typical for smooth-reacting types.

12. A positive acriflavine slide-reaction typical for nonsmooth-reacting types.

The incorporation into solid media of 2,3,5,-triphenyl tetrazolium chloride in a concentration of 1:125,000 was found to aid somewhat in the differentiation between colony types by producing striking color differences between colony type variants.

Isolation of new colony type variants and their origin. Following the recognition and isolation of colonial variants in stock cultures, additional variant types were obtained by growing smooth clones of Schu or 38 in peptone cysteine broth without agitation, at 37 C for prolonged periods. Periodic sampling of these cultures revealed that following 4 days of incubation of 38 (S), a progressive estab-

TABLE 1

Effect of initial pH and size of inoculum upon the establishment of nonsmooth variants in originally smooth cultures of Bacterium tularense

DAYS' INCUBATION AT 37 C	INITIAL pH*	INITIAL VIABLE CELL INOCULUM PER ML	pH AT TIME OF SAMPLING	VIABLE CELLS PER ML	PACKED CELL VOLUME PERCENTAGE	PERCENTAGE NONSMOOTH VARIANTS
0	6.8	120,000	6.8	120,000	—	0
	7.2	120,000	7.2	120,000	—	0
	6.8	130,000,000	6.8	130,000,000	—	0
	7.2	130,000,000	7.2	130,000,000	—	0
2	6.8	120,000	6.9	710,000,000	0.05	2
	7.2	120,000	7.4	670,000,000	0.04	0
	6.8	130,000,000	7.0	1,200,000,000†	0.07	23
	7.2	130,000,000	7.45	1,000,000,000	0.07	0
4	6.8	120,000	7.1	970,000,000†	0.13	16
	7.2	120,000	7.5	1,000,000,000	0.09	0
	6.8	130,000,000	7.1	1,500,000,000†	0.17	52
	7.2	130,000,000	7.5	980,000,000	0.14	0
8	6.8	120,000	7.1	1,400,000,000†	0.22	65
	7.2	120,000	7.5	1,300,000,000	0.18	0
	6.8	130,000,000	7.1	1,500,000,000†	0.24	64
	7.2	130,000,000	7.5	1,100,000,000	0.20	0

* Determined after sterilization.

† Note that a considerable percentage of these viable cells is represented by nonsmooth types.

lishment of nonsmooth colonial types took place. Despite its faster growth rate, the Schu strain did not show significant numbers of nonsmooth variants until 8 days of incubation. Subsequent studies, summarized in table 1, revealed that the rate at which such population changes occurred depended greatly on the inoculum size and the initial pH. It may be seen that cultures inoculated with a small number of cells show much less rapid population changes during the first 4 days than do similar cultures inoculated with approximately 1,000 times as many cells. A complete absence of population changes in cultures initiated at pH 7.2 was noted during the 8-day period of observation, whereas similar cul-

tures initiated at pH 6.8 displayed as high as 65 per cent variant types after 8 days. It was also observed that, under otherwise identical environmental conditions, different lots of peptone used in the preparation of the culture medium may significantly modify the rate of population changes. These observations are similar to those made in studies on *Brucella abortus* (Braun, 1946). They support the conclusions derived from the earlier study, namely, that the results were compatible with the interpretation that population changes are due to the progressive establishment of spontaneously arising variants (mutants) that are better adapted to the environmental conditions existing in the cultures after the initial maximum increase in viable cells of the original type. The data in table 1 illustrate that the establishment of nonsmooth variants occurs following the cessation of increase in number of viable smooth cells and during continued increase in the total number of cells, as previously demonstrated for *Brucella*.

The reduction of population changes at pH 7.2 compared to cultures initiated at pH 6.8 could be due to reduced growth, which would provide less opportunity for the creation of competitive conditions favoring the establishment of nonsmooth variants, but is more probably due to a modification of metabolite production in this pH range. The manner in which metabolites may be involved in population changes can best be indicated by reference to recent results with *Brucella* (Goodlow, Mika, and Braun, 1950). These studies demonstrated that in originally smooth cultures a cessation of increase in the number of viable smooth cells and the establishment of nonsmooth variants coincided with the accumulation of a toxic metabolite, alanine, in the culture medium. It was subsequently determined that alanine accumulation was one of the factors responsible for population changes and that those variants which established themselves progressively in aging cultures exhibited increased alanine resistance.

Attempts have been made to determine whether the accumulation of this amino acid may also be responsible for population changes of *B. tularense*. Accordingly, the basal liquid medium was supplemented with either 100, 500, 1,000, or 2,000 μg of DL-alanine per ml. Cultures seeded with either smooth Schu S_1 or smooth Schu S_3 clones were then sampled at various intervals during incubation at 37 C. No differences in viable count, total count, or percentage of variants could be detected between control cultures and alanine-supplemented cultures. However, when similar experiments were performed with filtrates of old cultures, a significant effect upon population changes was observed. In these experiments the basal liquid medium was supplemented with sterile filtrates from 8-day-old cultures originally inoculated with Schu S_1 and containing 50 per cent nonsmooth types at time of filtration through sintered glass filters (porosity UF). Either 0.1-ml or 1.0-ml amounts of sterile filtrate were added to 4.9 ml or 4.0 ml of basal liquid medium. All media were then seeded with 5.0×10^8 cells of a smooth Schu S_1 clone. Data presented in table 2 reveal a significantly faster establishment of nonsmooth variants in cultures containing the lesser amount of filtrate than in cultures containing no filtrate. The reduced intensity of this effect in cultures supplemented with the greater amount of filtrate may have been due to a toxic effect of such severity that growth of both smooth and

nonsmooth types was sufficiently suppressed to prevent opportunities for population changes. The responsible factor or factors accumulating in the old cultures have not been determined as yet. Nevertheless, it appears that population changes in *B. tularense* may be due to the accumulation of a metabolite that is toxic for the parent type but is of lesser toxicity for spontaneously occurring metabolite-resistant mutants.

Population changes from S → NS → S have been noted during prolonged growth in liquid media. These changes were accompanied by a significant reduction of virulence characteristics of the NS type and a restoration of partial or full virulence of the S type obtained after aging of the NS culture. Despite the similarities in colony type and virulence characteristics of the original S type and the one obtained after aging of NS cultures, such changes probably represent changes from S → NS → S′, rather than changes from S ⇄ NS, where S and S′ differ in some important respects. In similar studies with *Brucella* (Goodlow, Mika, and Braun, 1950), it was determined that despite similarities in colony type, antigenicity, and virulence, there existed a significant difference

TABLE 2

Influence of old culture filtrates upon the variation of Bacterium tularense during growth in liquid medium

PERCENTAGE STERILE 8-DAY FILTRATE ADDED TO PEPTONE CYSTEINE BROTH	PERCENTAGE NONSMOOTH VARIANTS				
	Days' incubation at 37 C				
	0	1	2	3	4
2	0	0	0	9	54
20	0	0	0	8	7
0 (control)	0	0	0	0	6

in the alanine resistance of S and S′ types. The former was highly susceptible to the toxicity of the accumulating metabolite, whereas the latter was much more resistant to the toxic effect. In view of the indication that the accumulation of certain toxic metabolites may also play a critical role in population changes of *B. tularense*, it can be assumed that similar differences may exist in the characteristics of S and S′ of *B. tularense*.

In contrast to past experience with the *Brucella* species, when such population changes could only be detected following growth in liquid media, it was found that certain variants of *B. tularense* gave rise to population changes following frequent serial transfers and prolonged incubation on solid media. However, the majority of variants isolated after growth in liquid media or frequent transfers on solid media gave rise to stable subcultures if the latter were maintained on slants stored at 5 C following incubation for 24 hours at 37 C.

Attempts to investigate population changes in buffered cultures have been unsuccessful because of the toxic effects of the buffers employed (K_2HPO_4 or citric acid + Na_2HPO_4).

Uranium acetate, which has been reported to increase variation in fungi

(Stakman *et al.*, 1948), was found to enhance greatly population changes in *B. tularense*. Broth cultures of Schu S_3 to which 0.01 per cent uranium acetate had been added showed 80 to 90 per cent nonsmooth variants after 5 days of incubation, whereas cultures without uranium acetate showed no variants after the same period. It has not yet been determined whether this effect is due to a selective or a mutagenic activity of the uranium salt.

Acriflavine tests. The acriflavine test, which for the *Brucella* species had proved to be a reliable indicator of antigenic characteristics (Braun and Bonestell, 1947), has been applied to the colony type variants of *B. tularense*. It had been found that antigenic characteristics of the *Brucella* species may occasionally vary independently of changes in colony morphology. The acriflavine test had made it possible to detect such independent variation. A similar independent variation

TABLE 3

Properties of certain Bacterium tularense variants

VARIANT DESIGNATION	COLONIAL APPEARANCE	ACRIFLAVINE AGGLUTINATION	ACID AGGLUTINATION RANGE (pH)	LD_{100} FOR MICE (ORGANISMS)
Schu S_1	Smooth—buff	0	No agglutination*	1–10
Schu S_2	Smooth—buff	0	No agglutination*	1–10
Schu S_3	Smooth with blue ring	0	No agglutination*	1–10
Schu S_4	Smooth—blue	0	No agglutination*	1–10
Schu NS^s	Nonsmooth with central plateau	0	No agglutination*	1–10
Schu NS_2	Nonsmooth—gray	+	2.6–5.0	10 million
Schu NS_3	Nonsmooth—gray	+	2.6–5.0	100 million
38 S_1	Smooth with blue ring	0	No agglutination*	Avirulent
38 S_2^{ns}	Smooth—Blue	+	2.2–3.8	Avirulent
38 NS_1	Nonsmooth—buff	+	2.6–4.2	Avirulent
38 S_2^{ns}	Smooth—gray	+	2.2–4.2	Avirulent
38 NS_2	Nonsmooth—gray	+	2.6–4.2	Avirulent
38 NS_d	Nonsmooth—dwarf	+	2.6–4.2	Avirulent

* pH 2.2 to 7.0.

between colony morphology and acriflavine reactions has been observed in *B. tularense*. The test was employed in either of two ways: (1) by suspending cells in saline and observing the suspension macroscopically for the presence or absence of flocculation after the addition of an equal volume of 1:500 acriflavine, or (2) by emulsifying a single colony in a drop of saline on a glass slide and observing the results microscopically after the addition of a drop of a 1:1,000 solution of neutral acriflavine. Two distinctly different reactions were observed with either test: one in which the suspension remained uniformly turbid and another in which a precipitate appeared (see figure 1, nos. 11 and 12).

It was observed that not all smooth-appearing types of strain 38 tested were not agglutinated by acriflavine. All nonsmooth-appearing colony types, except one, were agglutinated (table 3). The exceptional nonsmooth-appearing type (figure 1, no. 6) yielded a smooth acriflavine reaction and in subsequent viru-

lence titration proved to possess virulence similar to its smooth-appearing parent type. This exceptional type may, therefore, serve as an example of the occasional occurrence of independent variation between colony morphology and acriflavine reaction, and it demonstrates that colony type observations alone are not sufficient for the reliable classification of variants.

Practically all nonsmooth types of *B. tularense* strains tested yielded an acriflavine reaction like that of mucoid types of other species. No reason for the paucity of rough-reacting types of *B. tularense* has been discovered as yet.

Because independent variation between colony morphology and acriflavine reactions occurs rather frequently, the terms *smooth-reacting* or *nonsmooth-reacting* will be used subsequently to denote the results of the acriflavine reaction.

Acid agglutination and behavior in saline. The results of the acriflavine reaction were found to be directly correlated with the results of acid agglutination tests (table 3).

The tendency of most nonsmooth variants to agglutinate in saline appears to explain the simple and efficient separation of smooth types from mixed populations in U-tubes containing a sintered glass filter disc at the base (Braun and Howell, 1950). It was found that, when a mixture containing as much as 90 per cent nonsmooth and 10 per cent smooth (Schu) types was inoculated into one side of such a U-tube containing broth, smooth types only could be isolated from the uninoculated side 24 to 48 hours later.

Virulence of colony type variants. Virulence titrations of smooth clones of Schu and 38 (isolated from single colonies), as well as of colony type variants derived from these clones, were performed using the mouse as the test animal.

Mice weighing approximately 20 g were inoculated intraperitoneally with 0.5 ml of serial 10-fold dilutions of a 0.1 per cent gelatin, 0.85 per cent saline suspension of cells obtained from 24-hour GCBA slants. For these experiments the minimum number of cells required to kill 100 per cent of the inoculated mice within 15 days (LD_{100}) has been used as an indicator of virulence. The results, obtained with a total of 520 mice and summarized in table 3, demonstrate that 1 to 10 organisms of all smooth-reacting Schu variants are capable of killing mice. The nonsmooth-reacting variants of Schu, however, killed mice only if administered in millionfold doses. As expected, both smooth and nonsmooth variants of strain 38 proved to be avirulent. The general avirulence of all strain 38 variants may serve as additional evidence of the occasional occurrence of independent variation between virulence, colony morphology, and results of the acriflavine test. In this respect the general avirulence of smooth and nonsmooth types of strain 38 of *B. tularense* is very similar to the greatly reduced virulence of strain 19 of *B. abortus* (Braun, 1950).

In addition, the virulence of three smooth- and one nonsmooth-reacting colony type variants obtained from a culture designated Jap was determined. This culture, generally considered to be of low virulence (Downs and Woodward, 1949), was originally isolated from a human lymph node in 1926 (cf. Hesselbrock and Foshay, 1945) and, when colony types were inspected, was found to contain two smooth-reacting

types, which were designated Jap S_1 and Jap S_2. Upon aging in broth for 5 days, two additional colony types, one smooth-reacting and another nonsmooth-reacting, were recovered and designated Jap S_3 and Jap S_4^{ns}. In virulence titrations with mice it was found that only Jap S_2 showed virulence similar to that of the heterogeneous parent culture, whereas the other three types exhibited markedly less virulence (table 4). The lower virulence of the smooth-reacting types Jap S_1 and Jap S_3 further exemplifies the occasional independent variation between virulence characteristics and results of the acriflavine test.

In contrast to the experience with *Brucella*, the frequent occurrence in *B. tularense* of significantly different colony types that exhibit smooth acriflavine reactions and that retain full virulence is noteworthy. In studies of *Brucella* practically all variants deviating in appearance from the typical smooth type have been found to possess reduced virulence. Because the possibility remained that differences in virulence between the various smooth-reacting colony types of *B. tularense* may actually exist but may not be detectable when highly sus-

TABLE 4

Immunogenic potency of colony type variants obtained from Bacterium tularense strain Jap

Colony type used for preparation of living vaccine*			Number of animals (mice)	Percentage survival of mice challenged intraperitoneally with 1,000 cells of smooth Schu S_1
Designation	Mouse intraperitoneal LD_{100} (organisms)	Acriflavine reaction		
Jap (parent)	1.0×10^6	0	10	50
Jap S_1	1.0×10^9	0	10	0
Jap S_2	1.0×10^6	0	10	50
Jap S_3	1.0×10^8	0	10	0
Jap S_4^{ns}	$>1.0 \times 10^9$	+	10	0
Nonvaccinated controls			10	0

* Mice were given a single sublethal subcutaneous injection of 1×10^4 viable cells, and survivors were challenged 14 days thereafter.

ceptible mice are used as hosts, virulence titrations of some of the smooth Schu variants were also performed with more resistant rats. Although approximately 10 to 100 cells of these smooth-reacting types were required to kill rats, no significant differences in virulence were noticeable between the different colony types tested.

Immunogenic potency of colony types. The antigenicity of colony type variants of *B. tularense* was determined by testing in mice and rats the immunogenic potency of vaccines prepared from different clones. Phenol-killed vaccines were injected subcutaneously, and all mice were challenged with various intraperitoneal doses of a virulent smooth clone of Schu 2 weeks after vaccination.

Preliminary experiments indicated that all of the smooth-reacting colony types of Schu listed in table 2 were capable of producing equally good protection when phenol-killed vaccines of these types were tested in rats. All vaccinated rats (216 animals) received 15 billion killed cells per animal over a period of 3 days, and were subsequently challenged by the intraperitoneal route with 20, 2,000,

or 200,000 virulent cells per animal. The average LD_{50} for vaccinated animals was found to be approximately 100,000 organisms; the LD_{50} for nonvaccinated animals was 20 organisms. As yet, the immunogenic potency of nonsmooth-reacting types of Schu has not been determined.

Subsequently, living vaccines of strain 38 variants were tested in white rats by inoculating each animal subcutaneously with 15 billion living cells from one of the clones listed in table 3. The vaccination schedule and challenge doses were identical with those used in the Schu experiment. Results from a total of 288 animals indicated that protection was produced by both the smooth and nonsmooth types, increasing the average LD_{50} to approximately 100,000 organisms, compared to the LD_{50} of 20 organisms for the nonvaccinated controls. The ability of nonsmooth-reacting variants of strain 38 to produce efficient protection in rats may be ascribed to the presence of a small amount of immunogenic antigen in the nonsmooth types which is capable of elevating the relatively high level of natural resistance of rats sufficiently to produce detectable protective effects. In contrast, the level of natural resistance in mice is extremely low, and the small amount of immunogenic antigen in nonsmooth types may not suffice to elevate resistance to critical levels. This is further indicated by the difficulties encountered in producing significant protection in mice even with the smooth-reacting variants of strain 38, and by the demonstration that mice could be significantly protected by vaccination with certain smooth-reacting but not with nonsmooth-reacting variants obtained from the Jap culture.

Downs and Woodward (1949) reported previously that efficient protection of mice can be obtained when living cells of strain Jap are used as a vaccine. Each of the four different colony types isolated from the heterogeneous Jap culture and described in table 4 was used for the preparation of live vaccines. The results of experiments in which mice were vaccinated with these Jap strains are summarized in table 4. It may be seen that considerable differences in immunogenic potency of these four types could be detected. Protection equal to that produced by the heterogeneous parent culture was effected only by cells of the smooth-reacting Jap S_2 type; the other three types failed to protect mice that were challenged with 100 or 1,000 viable cells of smooth Schu S_1. The results of this experiment thus illustrate the existence of differences in immunogenic properties between variants of *B. tularense* and indicate a close relationship between the virulence and immunogenic potency of all variants so far tested.

DISCUSSION

The foregoing data clearly illustrate that changes in virulence and immunogenic properties of *B. tularense* are frequently linked with changes in colony type. The basic mechanism underlying these changes appears to involve spontaneous occurrence of undirected variants (mutants) in growing populations, and their subsequent establishment under environmental conditions that are more favorable for the variant types than for the parent type. (See detailed discussions by Braun, 1946, 1947.) Conditions adverse to the parent type are reached in all growing cultures and are apparently due to the accumulation of

one or more toxic factors. Indicators of toxicity are: (1) the cessation of increase in number of viable smooth cells, (2) the fact that, in studies with clones, variants do not establish themselves to a detectable extent until the increase in viable cells of the parent types levels off, and (3) the enhancement of the establishment of variant types (population changes) by the addition of filtrates from old cultures. The accumulating toxic factor or factors are as yet unknown, and they appear not to be alanine, which has been found to be one of the critical environmental factors affecting population changes of *B. abortus*. The rate at which population changes occur depends greatly on inherent and environmental factors. Different *B. tularense* strains under identical environmental conditions exhibit significantly different tendencies toward population changes. The differences may be due to inherently different growth rates as well as to differences in rate of production of toxic factors. Thus, after equal growth periods, the percentages of variants (dissociation percentages) in cultures of strain 38 of *B. tularense* are consistently higher than in cultures of strain Schu maintained under identical conditions. These differences do not necessarily imply different mutation rates for these two strains; a mechanism that permits the more rapid attainment of cultural conditions adverse to the parent type, e.g., more rapid toxic metabolite production by strain 38, could account for these differences despite equal mutation rates.

Little is known as yet concerning the actual mutation rates. In conformance with known mutation rates in other micoorganisms, it may be assumed that the rate is low, approximately 1×10^{-8} mutations per bacterium per time unit. However, recent studies have provided means for actual measurement of mutation rates in *B. tularense*. Unpublished studies on cultures containing unfiltered normal serum have shown that the establishment of nonsmooth-reacting types of *B. tularense* is entirely inhibited in the presence of 5 per cent serum. This will permit measurement of mutation rates from NS $\rightarrow$ S′ with the help of the fluctuation test (Luria and Delbruck, 1943) employing media that will selectively suppress the parent type but allow mutants to grow.

It has been demonstrated that environmental conditions that will modify population dynamics will also modify the dissociation percentage. Thus, the initial pH of the cultures and inoculum size have a significant effect. In addition it has been recognized that there will be far less opportunity for the establishment of variant types in shaken or otherwise aerated cultures. At the present time this latter effect can be best explained on the assumption that it involves the oxidative removal of toxic metabolites concerned in population changes.

The demonstration of considerable heterogeneity in what had been assumed to be pure stock cultures and the recognition of variation in important characteristics emphasize the importance of using clones and of controlling the factors that cause population changes in experimental studies with *B. tularense*. Without recognition of these factors, certain cultures may be assumed to possess innate differences in virulence (or immunogenic properties), whereas they may merely represent related heterogeneous populations containing different proportions of highly virulent and weakly virulent types. The analysis of the virulence

and immunogenic potency of different colony types isolated from strain Jap may serve as an example.

Previously noted gradual loss of virulence in laboratory-maintained cultures can now be easily understood on the basis of the experimentally demonstrated progressive population changes from smooth to nonsmooth. Similarly, previous reports on the increase in virulence of cultures following passage through animals can now be explained on the basis of *in vivo* selection of highly virulent types from originally heterogeneous populations. It has been observed that, as in *Brucella* (Braun, 1949), specific host factors, which suppress the *in vitro* establishment of less virulent nonsmooth types, exist in the serum of susceptible animals. These effects of sera upon specific types and population changes of *B. tularense* will be reported separately in a subsequent publication.

SUMMARY

The recognition of colony type variants of *Bacterium tularense* has been made possible by the use of an oblique lighting technique in the examination of colonies on transparent agar medium. It has been demonstrated that changes in virulence and immunogenic properties of *Bacterium tularense* are frequently associated with an alteration of colony types. Changes in these properties are most frequently observed following prolonged incubation in liquid media and can be attributed to the progressive establishment of spontaneously arising variants (mutants) in growing cultures under conditions that are more favorable for the variant type than for the parent type. The type and composition of the medium, the initial pH, and the inoculum size affect population changes, and one or more factors present in old culture filtrates appear to play an important role in the creation of environmental conditions that lead to population changes. Correlations have been made between the acriflavine reaction, acid agglutination, virulence, immunogenic potency, and the morphology of various colony types isolated from a variety of *Bacterium tularense* cultures. The relation of these observations to general problems encountered in studies with *Bacterium tularense* has been discussed.

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