STUDIES ON TULAREMIA

I. The Relation Between Certain Pathogenic and Immunogenic Properties of Variants of *Pasteurella tularensis*

MAX D. MOODY[1, 2] AND CORA M. DOWNS

Department of Bacteriology, University of Kansas, Lawrence, Kansas

Received for publication March 7, 1955

Many authors have concerned themselves with the relation between virulence and immunogenicity in numerous species of organisms. The relation between the property of virulence and that of immunogenicity, and the relation of these properties to variation in *Pasteurella tularensis* are the subject of the following papers. Data are presented which demonstrate the degree of correlation which may be made among the following properties of the organisms used: (1) the colonial characteristics, (2) the toxicity of large numbers of living organisms in the white mouse, (3) the ability to proliferate in the white mouse, and (4) the immunogenicity for the white mouse.

Considerable variation among pathogenic and immunogenic properties of strains of *P. tularensis* have been reported in the literature. This variation has occurred both in nature and in the laboratory (Francis, 1922; Green, 1943; Foshay, 1932; Phillip and Davis, 1935; Ransmeier, 1943). Many attempts have been made to determine the relationships existing between such properties as virulence, immunogenicity, and morphology using various species of animals. Substantial evidence has been reported, however, which indicates that immunogenic potency is contingent upon the ability of a strain to invade the tissues and rapidly multiply. It has been suggested that, during this rapid early multiplication in the host, a protective immunogenic antigen is released, resulting in the establishment of protective antibody (Downs *et al.*, 1949; Downs *et al.*, 1947*a*; Downs and Woodward, 1949).

It was felt that by the use of variants of *P. tularensis* strains, more exact information could be obtained, and thereby the relationships existing among certain properties would be more clearly defined. During the progress of the studies Eigelsbach *et al.* (1951) reported recognition of various colony types of *P. tularensis*. Some correlations were made between the acriflavine reaction, acid agglutination, virulence, immunogenic power, and the morphology of colony types isolated from various strains. In 1952 Eigelsbach *et al.* reported differences in protective ability of colony type variants as well as their ability to survive in the host.

Our present studies, using variants of *P. tularensis*, are therefore a continuation of the work done in this laboratory and cited above. In the present paper, data are reported which clearly demonstrate that organisms of variants which possess a high degree of immunogenic potency do proliferate freely, are toxic, and are smooth in colonial characteristics.

MATERIALS AND METHODS

Strains of P. tularensis. The parent strains used in these experiments possessed various known characteristics, some of which have been previously reported. The 38 strain was selected as an avirulent strain which has been shown to lack immunogenicity and the ability to multiply in white mice. The Jap and Ri parent strains are of moderate virulence, they are immunogenic, and they multiply in the white mouse. Sm was selected as a fully virulent culture. It proliferates rapidly in mice and immunizes if streptomycin therapy is used to prevent death of the mice after infection.

Media. Variants were isolated from the above cultures grown in Snyder's peptone broth containing 1 per cent dextrose (Snyder *et al.*, 1946*b*). The pH of the broth was adjusted to 6.8. All plate counts were made on glucose-cysteine-blood

[1] From a thesis submitted in partial fulfillment for the requirements of the degree of Doctor of philosophy, University of Kansas, Lawrence.

[2] Present address: Communicable Disease Center, Laboratory Branch, P. O. Box 185, Chamblee, Georgia.

agar (GCBA) (Downs *et al.*, 1947*a*). Colonial morphology was observed on GCBA and on Snyder's agar (Snyder *et al.*, 1946*a*), which contained 1 per cent dextrose.

Preparation of inocula. In all cases the original inoculum used for cultures or animal infection was prepared by emulsifying in physiological saline the growth from a GCBA slant which had incubated 24 hours at 37 C. The turbidity was adjusted to give 24 per cent light transmittance using a Coleman No. 11 Universal spectrophotometer set at a wavelength of 600 micra. This is considered as a standard suspension which usually contains 2 to 4 billion organisms per ml. Serial 10-fold dilutions were made in sterile 9 ml physiological saline blanks. Plate counts were made in triplicate by plating 0.1 ml of the 10^{-6} and 10^{-7} dilutions per plate on GCBA. Further details of the techniques used are indicated in the separate experiments.

Establishment of variants from parent strains. Variants were established from Snyder's broth cultures which had been inoculated with the respective parent strains and incubated at 37 C for 5 to 10 day periods. These cultures were streaked with a wire loop on GCBA and on Snyder's agar and incubated 72 hours at 37 C. Colonies were observed with a dissecting microscope and oblique light transmittance (Henry 1933). The method of White and Wilson (1951) for examining Brucella colonies was successfully applied in examining colonies of *P. tularensis* in these experiments. The consistency of representative colonies was tested by stabbing with a platinum wire. Those with differences in consistency were tested with acriflavine using the slide method (Braun and Bonestell, 1947). Various colonial types were transferred to GCBA slants and maintained as stock cultures at 0 to 4 C after incubation for 24 hours at 37 C. Transfers to fresh slants were made at 6 to 8 week intervals.

Testing of saline sensitivity. Saline sensitivity was determined by incubating at 37 C overnight a standard suspension of the living cells prepared in 0.85 per cent sodium chloride. Precipitation in the tubes indicated saline sensitivity.

Determination of virulence. The LD_{50} was determined by injecting intraperitoneally 0.5 ml of 10-fold serial dilutions of standard suspensions into 6 to 10 mice per dilution. The calculations were made according to the method of Reed and Muench (1938).

Further details of the techniques used are indicated in the separate experiments.

RESULTS

Comparison of virulence of variants with morphology, acriflavine reaction, and saline sensitivity. The differences in colonial morphology of the variants on both GCBA and Snyder's agar plates was well defined in most cases. Until the crystal violet method was used, however, colonial differences could not be consistently detected on GCBA. In our hands the observation of colonies of *P. tularensis* on Synder's agar as described by Eigelsbach *et al.* (1951) without the treatment with crystal violet was not so satisfactory as when crystal violet was used. In the majority of cases the appearance of the colonies on GCBA compared well with their appearance on Snyder's agar. The consistency and acriflavine reactions were compared before and after treatment with crystal violet and were found to agree. Smooth, buttery, acriflavine nonreacting colonies appear pinkish-violet to yellow, whereas mucoid or rough colonies took on a deep, uniform reddish-purple. It is imperative, in order to obtain the most satisfactory results, that only well isolated, well developed colonies be described.

The results of these findings and the original source of the parent strains are recorded in table 1. The table shows a definite correlation between the colony type, acriflavine reaction, and saline sensitivity of the variants. Each nonsmooth culture, for example, gave a positive acriflavine reaction and was saline sensitive. In the case of the parent strains, the failure to give a positive acriflavine reaction and to be saline sensitive may be attributed to the fact that the predominant colonial type was smooth in each case prior to being grown for extended periods in broth. Under normal conditions of growth on artificial media, occasional nonsmooth forms are seen; therefore, the colonial type of the parent strains is designated "S-NS". Although the usual change on repeated transfer on culture media is from smooth to nonsmooth, the reverse change occasionally occurs. This was true of the Ri_3 variant which changed from rough to smooth after 12 to 15 subcultures. This change was accompanied by an increased ability of the strain to multiply in the mouse, increased ability to immunize, and increased virulence for mice.

Table 1 shows that, though a culture may be

TABLE 1

Source and reactions of Pasteurella tularensis cultures

Culture	Geographic Source	Year of Isolation	Specific Source	Mouse LD_{50}	Colony Type	Acriflavine	Saline
38*	Utah	1920	Human lymph node	10^{0}	S-NS	0	0
38_1	KU†	1950	Parent strain	10^{0}	NS	+	+
38_2	KU	1950	Parent strain	10^{0}	S	0	0
38_3	KU	1950	Parent strain	10^{0}	S	0	0
38_4	KU	1950	Parent strain	10^{0}	NS	+	+
Jap*	Japan	1926	Human lymph node	$10^{-4.4}$	S-NS	0	0
Jap_1	KU	1950	Parent strain	$10^{-2.4}$	S	0	0
Jap_2	KU	1950	Parent strain	10^{0}	NS	+	+
Jap_3	CD		Parent strain	$10^{-4.3}$	S	0	0
Jap_4	CD‡		Parent strain	$10^{-0.7}$	S	0	0
Jap_5	CD		Parent strain	$10^{-1.1}$	NS	+	+
Jap_6	CD		Parent strain	$10^{-0.5}$	S	0	0
Ri*	Virginia	1932	Human pus	$10^{-6.1}$	S-NS	0	0
Ri_1	KU	1950	Parent strain	$10^{-6.1}$	S	0	0
Ri_2	KU	1950	Parent strain	$10^{-0.5}$	NS	+	+
Ri_3	KU	1950	Parent strain	$10^{-2.6}$	NS	+	+
Ri_4	KU	1950	Parent strain	$10^{-5.0}$	S	0	0
Sm*	Ohio	1941	Human ulcer	$10^{-9.5}$	S-NS	0	0
Sm_1	KU	1950	Parent strain	$10^{-9.3}$	S	0	0
Sm_2	KU	1950	Parent strain	$10^{-3.5}$	NS	+	+
Sm A	CD		Parent strain	$10^{-9.6}$	S	0	0
Sm O	CD		Parent strain	$10^{-2.2}$	NS	+	+

* Parent strain.

† KU = isolated at the University of Kansas.

‡ CD = obtained from Dr. H. T. Eigelsbach of Camp Detrick.

§ No deaths resulted when 0.5 ml containing 2 to 4 billion organisms per ml were injected intraperitoneally.

smooth, may give a negative acriflavine reaction, and is not saline sensitive, it will not necessarily be virulent when tested in mice. Cultures having an LD_{50} of $10^{-3.5}$ or below may be either smooth or nonsmooth. On the other hand, the data do indicate that all cultures having a mouse LD_{50} above $10^{-4.3}$ are smooth or, in the case of parent strains, predominantly smooth.

No variants were isolated from a parent strain which were significantly more virulent than the parent strain itself. The fully virulent Sm strain and the Ri and Jap strains developed variants of significantly less virulence than the parent and of differing colonial morphology. From the 38 strain were isolated both smooth and nonsmooth variants, none of which were virulent for mice. In all the cultures used, absolute correlation between consistency and appearance of the colony, acriflavine reaction, and saline sensitivity existed.

The toxicity of cultures in white mice. The toxicity of the various cultures in white mice was determined by injecting large numbers of living organisms intraperitoneally. Downs, in unpublished experiments, has shown that large numbers of organisms of *P. tularensis* killed by various means are not toxic for white mice. In the present experiments at least 10 mice were injected with the variants, with the exception of the variants of the 38 strain. Each mouse was injected intraperitoneally with 0.5 ml of a standard suspension of living organisms (1 to 2 billion). The mice were observed at short intervals over a period of 24 hours, and the numbers of dead mice were recorded. The survivors were observed for 10 days after injection. The percentage survival and the average day of death was calculated. The average day of death was used as the criterion of toxicity. Arbitrarily, it is suggested that an average day of death under 2.0 be considered the result of toxicity.

The results shown in table 2, with the exception of cultures of the 38 strain, indicate that most cultures are reasonably toxic for mice, regardless

TABLE 2

The pathogenic and immunogenic properties of parent strains and variants of Pasteurella tularensis

Culture	Immunizing Dilution	Per Cent Surviving after Sm A Challenge Dose		Colonial Type	LD_{50}	Multiplication†	Toxicity‡
		100 LD_{50}	1000 LD_{50}				
Sm*	10^{-7}	100	—	S-NS	$10^{-9.5}$	94.1	1.2
Ri^1	10^{-7}	100	71	S	$10^{-6.1}$	84.7	1.1
Ri*	10^{-7}	80	100	S-NS	$10^{-6.1}$	78.2	.93
Ri_4	10^{-5}	100	100	S	$10^{-5.0}$	71.7	.92
	10^{-7}	100	88	S			
Jap_3	10^{-4}	100	100	S	$10^{-4.3}$	72.8	.83
	10^{-6}	100	100	S			
Sm_1	10^{-7}	80	55	S	$10^{-9.3}$	99.4	.98
Jap*	10^{-4}	83	92	S-NS	$10^{-4.4}$	69.6	.75
	10^{-5}	90	80				
Jap_4	10^{-2}	80	92	S	$10^{-0.7}$	37.7	1.4
	10^{-4}	70	60				
	10^{-6}	80	70				
Sm_2	10^{-4}	50	55	NS	$10^{-3.5}$	35.8	.95
Sm 0	10^{-3}	11	20	NS	$10^{-2.2}$	21.4	2.4
	10^{-6}	10	0				
Jap_5	10^{-2}	30	20	NS	$10^{-1.1}$	31.8	1.45
	10^{-4}	0	10				
	10^{-6}	0	0				
Ri_3	10^{-5}	16	25	NS	$10^{-2.6}$	0	1.3
	10^{-7}	0	0				
Ri_2	10^{-1}	30	30				
	10^{-2}	10	30				
	10^{-4}	0	0	NS	$10^{-0.5}$	0	2.6
	10^{-5}	0	0				
	10^{-7}	0	0				
Jap_6	10^{-2}	0	10	S	$10^{-0.5}$	38.4	1.0
	10^{-4}	0	0				
	10^{-6}	10	0				
38*	10^{-1}	23	0	S-NS	10^{0}	0	—
38_3	10^{0}	0	0	S	10^{0}	0	—
	10^{-2}	0	0				
38_4	10^{0}	0	0	NS	10^{0}	0	—
	10^{-2}	0	0				
Normal controls	—	0	0				

* Parent strain.

† Represents weight in milligrams of graph paper lying beneath the curve when logarithm of number of organisms obtained per gram of spleen was plotted at the various time intervals after injection of 1 to 2 thousand organisms.

‡ Figures represent the average day of death for mice injected with 1×10^9 organisms.

of their colonial morphology and virulence. For example, Sm_2, a nonsmooth and slightly virulent culture, was more toxic than were the smooth, fully virulent Sm A and Sm cultures. In most cases, however, the more virulent smooth cultures were more toxic than were the less virulent nonsmooth cultures. These data suggest that a relatively avirulent culture may be toxic when large numbers of living organisms are injected. Of interest is the fact that none of the avirulent strain 38 cultures killed the mice even when doses of 4 billion organisms were injected.

In one experiment the number of cells from tissues 18 hours after injection with toxic and

atoxic variants was determined. Although the number of mice tested could not be considered sufficient, it was observed that when comparable numbers of organisms were injected, comparable numbers were recovered at 18 hours, regardless of the strain injected. This observation, along with the fact that avirulent as well as virulent strains resulted in very rapid death, lends support to the contention that death was a result of toxicity rather than of multiplication in the animal.

The multiplication of organisms during infection. The relationship between immunogenicity and ability to multiply in the host, which was demonstrated for *P. tularensis* (Downs and Woodward, 1949) with living parent strains, suggested the necessity of making quantitative plate counts during infection with variants isolated from the parent cultures. Mice were injected intraperitoneally with approximately 1,000 to 2,000 organisms from a 24 hour slant culture. Two mice were sacrificed immediately after injection and at 24, 48, 72, and 96 hours. The spleen was weighed and ground in a mortar, and serial 10-fold dilutions were made in physiological saline and plated in duplicate on GCBA using 0.1 ml per plate. The plates were incubated 72 hours at 37 C and counted. The numbers of organisms recovered per gram of spleen from the two mice were averaged.

The results obtained are shown in table 2. The figures given were obtained by plotting the logarithm of the average number of organisms recovered per gram of spleen from the two mice at each time interval. In each case the variation observed between the mice was considered insignificant. The area of graph paper lying beneath the curve was cut out and weighed on an analytical balance. By comparing the weights corresponding with each culture a definite value for the various cultures in their ability to multiply could be determined. Seven cultures possessed a high value indicating extensive multiplication. Five cultures multiplied only moderately well, while five others multiplied little or not at all in the mice.

It may be observed that all cultures which multiplied well are smooth and have an LD_{50} of at least $10^{-4.4}$. Those which multiplied moderately well included both smooth and nonsmooth cultures; their LD_{50} values range from $10^{-0.5}$ to $10^{-3.5}$. Likewise, in the nonmultiplying cultures there are both smooth and nonsmooth types; the LD_{50} values in these cases range from 10^{0} to $10^{-2.6}$. Thus, among cultures which multiplied extensively, there is correlation between smoothness of colony and virulence. On the other hand, virulence and ability to multiply cannot be associated with morphology in cultures which do not proliferate significantly in the mouse.

In separate experiments with avirulent 38, 38_3, 38_4, and Ri_2, in which as many as 20 to 200 million organisms were injected intraperitoneally, the number of organisms cultured per gram of spleen during a 4 day infection period failed to show an increase, but gradually decreased instead.

The immunogenicity of the cultures in white mice. The observations made by Downs and Woodward (1949), that certain strains immunized well and others poorly, made it seem feasible to determine the immunogenic potency of several variants isolated from various parent cultures. Groups of 20 mice were injected with the dilutions of a standard suspension as indicated in table 2. The dilutions used were sublethal doses of the cultures, except in the cases of Sm and Sm_1, in which streptomycin therapy followed the initial inoculation in order to allow the mice to survive until challenged. Streptomycin therapy was given as follows. Each mouse received intraperitoneally 10,000 units of dihydrostreptomycin at 2 and at 3 days after injection and 2,000 units at 4 and 7 days.

Fourteen days after injection the groups of 20 mice were divided into two groups of 10 mice each. These were challenged with 100 and 1000 LD_{50} doses, respectively, of the fully virulent Sm A culture. At the same time 10 normal mice were injected similarly at each level. The mice were observed for 12 days and the per cent surviving was determined. Representative mice which died were autopsied; cultures of the heart blood and spleen were made as confirmation that tularemia was the cause of death.

Table 2 indicates that all the cultures from Sm to Jap_4, inclusive, possessed a very high degree of immunogenic potency. From Sm_2 down the list the ability to immunize gradually decreased. Among the less immunogenic cultures the degree of protection corresponded with the number of organisms initially injected. This is especially evident in the cases of Jap_5, Ri_3, and Ri_2, in which a slight amount of protection resulted following the injection of very large

numbers of organisms. It is interesting to observe that for cultures which possessed good immunizing qualities, as few as 100 organisms produce immunity against 1000 LD_{50} of a fully virulent strain. On the other hand, as many as 100 to 200 million organisms, in the case of some of the less immunogenic cultures, failed to protect the mice against a challenge of 100 LD_{50}. In fact, in an unreported experiment in which the parent 38 culture was tested by giving five daily injections which amounted to a total of approximately 58×10^9 organisms, only a very slight degree of protection resulted.

From the information included in table 2, it may be observed that the first six cultures listed possessed the highest degree of protective capacity, virulence, and proliferative ability, and all were predominantly smooth in colonial morphology. Jap_4 is an exception because it afforded reasonably good protection but was nearly avirulent, multiplied poorly, and was not particularly toxic. The remaining cultures were low in immunogenic potency, low in virulence, and multiplied poorly or not at all. With the exceptions of 38, 38_8 and Jap_6, they were nonsmooth cultures. Thus, the factors of immunogenic potency, virulence, and ability to multiply are correlated in the positive sense with smooth properties and in the negative sense with nonsmooth properties. Toxicity appeared to be almost entirely independent of the other properties.

DISCUSSION

The use of crystal violet in observing colonial morphology aided materially in the selection and identification of colonies in these studies. By its use either on GCBA or on Snyder's agar, colonies of different types may be easily differentiated. The acriflavine reaction was also employed in these experiments; but this reaction alone did not give as well defined differences between smooth and nonsmooth colonies as observation of crystal violet treated colonies. The consistency, appearance on agar, acriflavine reaction, and saline sensitivity showed close correlation among the variants that were established and tested in these studies. No nonsmooth colonies were isolated which showed full virulence, and in no case was the LD_{50} of the smooth variants higher than that for the parent strain. Eigelsbach *et al.* (1951) reported that occasionally smooth appearing colonies gave a positive acriflavine reaction and nonsmooth colonies were described which showed full virulence but did not agglutinate with acriflavine.

The relative instability of such properties as colonial characteristics, virulence, proliferative ability, and immunogenicity, which occur occasionally, was very apparent in the Jap_4, Jap_5, and Ri_8 variants. Such rapid changes as were observed are exceptions rather than the general rule, however, since the reported properties for the other cultures have remained stable for approximately two years. The change from rough to smooth for the Ri_8 variant, which was accompanied by increased virulence and increased ability to proliferate in and to immunize the white mouse, is the reverse of most changes which occur on artificial media. It indicates that dissociation is not always a degradation from a virulent to an avirulent culture. The conditions governing such changes are obscure, and Eigelsbach *et al.* (1951) point out that in their opinion the S forms which arise from N-S strains are not identical with the original S strain. Cultures isolated from human infections are usually fully virulent for mice, but many eventually dissociate to less virulent forms. The spontaneous and sometimes rapid loss of virulence has been described by Foshay (1932) and Ransmeier (1943) and was probably due to the overgrowth of nonsmooth forms which were favored by unknown environmental factors.

The parent strains studied here that possessed any virulence at all were made up of variants that differed in virulence, while no virulent variants were isolated from the completely avirulent 38 strain. Since the strain 38 lacked virulence, toxicity, and immunogenicity in both smooth and nonsmooth clones, it may be looked upon as a completely degraded strain. Similar findings were reported for *P. pestis* (Jawetz and Meyer, 1943) and implied for *P. tularensis* (Eigelsbach *et al.*, 1951).

It was found that toxicity manifested as an early death following injection of large numbers could not be correlated with the other properties tested. Variants which were the most virulent were very toxic, but not significantly more so than some of the almost completely avirulent cultures. None of the completely avirulent culture variants (38, 38_8, 38_4) were toxic. The nature of the toxicity is not known but appears to be similar to the toxicity of rickettsiae, since large numbers of living organisms are necessary to

demonstrate it. Downs, in unpublished experiments, has shown that large numbers of organisms killed by various means are not toxic for white mice.

The ability of a culture of *P. tularensis* to multiply in the host is definitely linked with smooth type colonial form and virulence. The cultures which were able to establish themselves and multiply abundantly during the first few days of infection were smooth and their virulence was such that not more than 50,000 organisms constituted an LD_{50}. Those that multiplied little or not at all were nonsmooth, with the exception of Jap_6, Jap_4, and 38_3, and were very low in virulence or completely avirulent. The relationship between virulence and ability to multiply in animals was reported for the parent cultures of Russ, Jap, and 38 (Downs *et al.*, 1947*a*, *b*; 1949; and Downs and Woodward, 1949). Similar relationships between virulence and ability to multiply are frequently cited in the literature. For example, in the case of tubercle bacilli, Middlebrook (1950) states that "the bacilli of the completely avirulent variant strains multiply little, if at all, even within the first few days after inoculation into susceptible hosts, in contrast to the unharnessed multiplication of virulent tubercle bacilli." Jawetz and Meyer (1944) found that the most marked characteristic of avirulent plague organisms was their inability to proliferate freely even in susceptible experimental animals. Data presented by Eigelsbach *et al.* (1952) indicate correlation between virulence and persistence of certain Jap variants *in vivo*.

Variants isolated from the Sm, Jap, and Ri strains differed widely in their ability to immunize mice. None of the variants from the 38 strain afforded protection against challenge with fully virulent Sm A organisms. The immunogenic potency was related to the ability of the culture to multiply and to its virulence. Smoothness in colonial morphology was associated with the more immunogenic cultures. With the exception of two variants (Jap_4 and Jap_6) those cultures which afforded very little or no protection were nonsmooth and almost completely degraded in virulence and did not multiply well in the white mouse. Only one case occurred in which virulence and immunogenicity did not correspond, that is, in Jap_4, which was smooth, multiplied only moderately well, had an LD_{50} of $10^{-0.7}$, but immunized to a fairly high degree. The situation here is similar to that in the case of *P. pestis* (Pollitzer, 1952). For example, the properties of the virulent immunogenic Sm strain of *P. tularensis* are similar to certain highly virulent immunogenic strains of *P. pestis*. The parent Jap strain and Jap_3 variant of *P. tularensis* are of reduced virulence, multiply freely in the mouse, and are highly immunogenic as is the E. V. strain of *P. pestis*. Another analogy is apparent in the case of the Jap_4 variant of *P. tularensis* and A1122 strain of *P. pestis*, both of which are of almost degraded virulence and do not multiply well in the mouse but are immunogenic for mice. In the case of *P. pestis* it has been well substantiated that the ability of a strain to immunize is associated with the persistence of envelope antigen in the tissues. The envelope substance may be introduced directly as a purified substance, as a portion of cells possessing large amounts, or may be formed as a result of multiplication of organisms, thereby producing the necessary amounts for protection to occur. The presence of a substance similar to the envelope substance of *P. pestis* has not been demonstrated for *P. tularensis*, but the possibility should not be overlooked.

These studies have demonstrated that certain correlations may be made among the pathogenic and immunogenic properties and colonial types of variants. All cultures with an LD_{50}, requiring 50,000 organisms or less, immunized mice when injected in sublethal doses, multiplied after injection, and gave smooth colonies.

Cultures requiring 200 million or more organisms for an LD_{50} were nonimmunogenic, multiplied little or not at all, and were predominantly nonsmooth in colonial type. Nevertheless, smooth colonies could be selected from the completely degraded strain 38 which did not multiply and which were nonimmunogenic. This seems to indicate that smooth colony type does not imply the presence of an immunogenic substance in the culture but may indicate that most organisms retaining the ability to produce smooth colonies also retain the ability to produce an immunogenic antigen *in vivo*.

ACKNOWLEDGMENTS

This work was supported in part by a contract between the Office of Naval Research, Department of the Navy, and the University of Kansas, and in part under Contract W-18-064-CM-235 between the University of Kansas and the

Biological Department, Chemical Corps, Camp Detrick, Maryland.

Grateful acknowledgment is also made for the skilled technical assistance of Mrs. Mary Jane Taylor and Mrs. Irene Dyke.

SUMMARY

Virulence, immunogenicity, and ability to multiply in the white mouse and smooth colonial morphology are closely associated.

Smooth and nonsmooth clones could be selected from all the parent strains tested and dissociation was favored in liquid media.

The toxicity of large numbers of organisms injected into mice could not be correlated with other properties.

The change from a nonsmooth to a smooth culture, accompanied by increased virulence and increased ability to multiply and to immunize the white mouse is reported for one variant.

The avirulent 38 strain yielded both smooth and nonsmooth colonial types but none were virulent, immunogenic, or toxic.

The use of crystal violet provides a simple means of identifying different colony types on glucose-cysteine-blood agar and on Snyder's peptone agar.

REFERENCES

BRAUN, W., AND BONESTELL, A. E. 1947 Independent variation of characteristics in *Brucella abortus* variants and their detection. Am. J. Vet. Research **8**, 386–390.

DOWNS, C. M., BUCHELE, L., AND EDGAR, E. P. 1949 Studies on pathogenesis and immunity in tularemia. I. The pathogenesis of tularemia in the white rat. J. Immunol., **63**, 117–133.

DOWNS, C. M., CORIELL, L. L., CHAPMAN, S. S., AND KLAUBER, A. 1947*a* The cultivation of *Bacterium tularense* in embryonated eggs. J. Bacteriol., **53**, 89–100.

DOWNS, C. M., CORIELL, L. L., EIGELSBACH, H. T., PLITT, K. F., PINCHOT, G. B., AND OWEN, B. J. 1947*b* Studies on tularemia. II. Immunization of white rats. J. Immunol., **56**, 229–243.

DOWNS, C. M., AND WOODWARD, J. M. 1949 Studies on pathogenesis and immunity in tularemia. III. Immunogenic properties for the white mouse of various strains of *Bacterium tularense*. J. Immunol., **63**, 147–163.

EIGELSBACH, H. T., BRAUN, W., AND HERRING, R. D. 1951 Studies on the variation of *Bacterium tularense*. J. Bacteriol., **61**, 557–569.

EIGELSBACH, H. T., BRAUN, W., AND HERRING, R. D. 1952 Studies on the immunogenic properties of *Bacterium tularense* variants. J. Infectious Diseases, **91**, 86–91.

FOSHAY, L. 1932 Induction of avirulence in *Pasteurella tularensis*. J. Infectious Diseases, **51**, 280–285.

FRANCIS, E. 1922 Tularemia. VI. Cultivation of *Bacterium tularense* on mediums new to this organism. Public Health Repts. (U.S.), **37**, 102–115.

GREEN, R. G. 1943 Virulence of tularemia as related to animal and arthropod hosts. Am. J. Hyg., **38**, 282–292.

HENRY, B. S. 1933 Dissociation in the genus Brucella. J. Infectious Diseases, **52**, 374–402.

JAWETZ, E., AND MEYER, K. F. 1943 Avirulent strains of *Pasteurella pestis*. J. Infectious Diseases, **73**, 124–143.

JAWETZ, E., AND MEYER, K. F. 1944 Experimental infection of the chick embryo with virulent and avirulent *Pasteurella pestis*. Am. J. Pathol., **20**, 457–469.

MIDDLEBROOK, G. 1950 The experimental analysis of virulence of tubercle bacilli. Bull. N. Y. Acad. Med., **26**, 498–506.

PHILLIP, C. B., AND DAVIS, G. E. 1935 Tularemia. Observations on a strain of low initial virulence from rabbit ticks. Public Health Repts. (U.S.), **50**, 909–911.

POLLITZER, R. 1952. Plague studies. 3. Problems in immunology. Bull. World Health Organization, **5**, 165–226.

RANSMEIER, J. C. 1943 The reaction of the chick embryo to virulent and non-virulent strains of *Bacterium tularense*. J. Infectious Diseases, **72**, 86–90.

REED, L. J., AND MUENCH, H. 1938 A simple method for estimating fifty per cent endpoints. Am. J. Hyg., **27**, 493–497.

SNYDER, T. L., ENGLEY, F. B. JR., PENFIELD, R. A., AND CREASY, J. C. 1946*a* A dilution plate count method for certain strains of *Bacterium tularense*. J. Bacteriol., **52**, 241–243.

SNYDER, T. L., PENFIELD, R. A., ENGLEY, F. B., JR., AND CREASY, J. C. 1946*b* Cultivation of *Bacterium tularense* in peptone media. Proc. Soc. Exptl. Biol. Med., **63**, 26–30.

WHITE, P. G., AND WILSON, J. B. 1951 Differentiation of smooth and non-smooth colonies of *Brucellae*. J. Bacteriol., **61**, 239–240.