STUDIES ON TULAREMIA

II. The Antigenic Properties of Variants of *Pasteurella tularensis* in Various Hosts

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In previous publications from our laboratory, we have shown that the immunizing ability of *Pasteurella tularensis* in rabbits, guinea pigs, mice, and rats varies with the species used. Rats may be readily immunized against virulent challenge by killed cultures of virulent or avirulent strains; rabbits and guinea pigs are poorly protected against virulent challenge by either killed cultures or by prior infection with cultures of lowered virulence. Mice, on the other hand, are well protected by infection with certain strains of low virulence. Recently, Bell *et al.* (1952), by the use of subcutaneous challenge with strains of less than full virulence, have shown that mice may be protected by vaccination with killed culture vaccines.

It has been demonstrated in our laboratory and in Eigelsbach's (1951, 1952) that parent strains may dissociate into smooth and nonsmooth colonial types. The smooth variants may or may not show full virulence for mice and other susceptible animals. We have shown that smooth, virulent, immunogenic strains; smooth, moderately virulent, immunogenic strains; and smooth, avirulent, but nonimmunogenic strains may be isolated from various parent cultures. Without exception, nonsmooth strains have been lacking in virulence and immunogenicity.

Because of the difficulty in immunizing susceptible animals with killed cultures, it is not clear whether the smooth, nonprotective, avirulent strains lack immunogenicity because they lack a protective antigen or because they fail to multiply *in vivo* and thus do not produce this protective antigen.

We have postulated that the poor immunogenicity of killed culture vaccines in mice is due to the lack of a labile antigen which may or may not be produced in culture media but which is produced during multiplication *in vivo*. Smooth clones of a fully degraded strain such as 38 are weakly immunogenic and also multiply poorly *in vivo*. The evidence, therefore, points to the necessity for multiplication of any strain which is fully protective *in vivo*. A very similar situation exists in the protective avirulent and nonprotective avirulent strains of *Pasteurella pestis*.

Serological studies by ourselves and others have failed to reveal the presence or absence of antigens associated with smoothness or immunogenicity, though Larson (1951) has recently reported that less heat stable antigen could be extracted from the avirulent 38 strain after ether extraction than from the virulent strains.

As a part of the work being carried on in our laboratory on an analysis of the antigens in *P. tularensis*, we have studied the relation of colonial morphology, the immunogenicity of living organisms, and the stimulation of circulating antibodies in susceptible and resistant hosts. The antibody response to a second dose of the variants was also determined, and finally the protection conferred by the variants was tested by challenge with virulent organisms.

MATERIALS AND METHODS

Cultures. A complete description of the history and properties of the cultures tested has been given in the previous paper (Moody and Downs, 1955); therefore, only a brief resumé of certain properties will be given here. The Ri_1 and Jap_3 variants and the parent Jap strain, (1) were smooth in colonial characteristics, (2) had a mouse LD_{50} above $10^{-4.3}$, that is, fewer than 200,000 organisms per mouse were required to kill 50 per cent of the mice injected, (3) multiplied well in mice, (4) protected mice when injected in sublethal doses, and (5) were toxic when in-

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jected in very large numbers. The Ri_2 and Jap_5 variants possessed properties as follows: (1) had nonsmooth colonial characteristics, (2) had mouse LD_{50} below $10^{-1.1}$, that is, required approximately 200×10^6 organisms per mouse to kill 50 per cent of the mice injected, (3) multiplied poorly in mice, (4) were nonimmunogenic in mice, and (5) were relatively atoxic for mice. The Jap_4 and Jap_6 variants are both smooth and nearly avirulent; however, Jap_4 multiplied and immunized better than did Jap_6. Recovery from infection with Jap_4 resulted in greater inhibition of multiplication of a virulent strain in challenge animals than did Jap_6.

Animals. The susceptible species of animals tested were white mice, guinea pigs, and rabbits; the naturally resistant species were chickens and white rats. No consideration of sex was made except in the case of chickens which were female. The mice were approximately 15 to 20 grams in weight and were obtained from the Maple Grove Rabbitry, Springfield, Missouri. The guinea pigs were healthy young adults supplied by the Endocrine Laboratory, University of Kansas. The rabbits weighed 4 to 6 pounds and were supplied by a local dealer. The chickens were of mixed breeds and weighed 3 to 5 pounds. The rats were of the Sprague Dawley strain and weighed approximately 100 grams.

Agglutination test antigens. The cell suspensions were prepared from cultures which had incubated 24 hours at 37 C. The organisms were suspended in saline, and formalin was added to give a final concentration of 0.25 per cent. After holding at 0 to 4 C overnight, the cells were collected by centrifugation and washed three times in 0.85 per cent saline. The suspensions at the time of testing were adjusted to contain approximately 2 to 4 billion organisms per ml.

Preparation of inocula for animals. Cultures were grown on glucose-cysteine-blood agar (GCBA) slants which were incubated 24 hours at 37 C. The organisms were emulsified in 0.85 per cent saline and adjusted to contain approximately 2 to 4 billion organisms per ml, as described previously. Serial tenfold dilutions of this suspension were made in saline. These dilutions were made as inocula for injecting the animals.

More specific details related to the individual experiments will be presented in the respective sections when necessary.

RESULTS

The disposal of variants of P. tularensis in normal and recovered mice after intravenous infection. It has been shown in the first paper of this series that the Jap_4 and Jap_6 variants were smooth and avirulent for white mice (Moody and Downs, 1955). The Jap_4 variant, however, was highly immunogenic, more toxic and multiplied slightly better in the mouse than did the Jap_6 variant. Recovery from the Jap_6 variant conferred little immunity to challenge with a virulent strain. It was suggested that immunogenicity is a function of multiplication in the host tissues rather than the presence of a smooth antigen. The present experiments were done to determine when and where the effective period of multiplication occurs in the animal, using Jap_4 as an example of the immunogenic strain and Jap_6 as the nonimmunogenic strain. The multiplication of fully virulent organisms in mice which had recovered from Jap_4 and Jap_6 infection was also determined. Normal mice injected with the virulent strain were used as controls.

Groups of mice were injected intravenously with approximately 1.2×10^6 organisms of the Jap_4 and Jap_6 variants, respectively. One group per variant was also injected intraperitoneally with similar dosages. The intravenously injected mice were weighed and sacrificed, two per group, at intervals between 4 and 96 hours after injection. Dilutions of the heart blood were plated on GCBA. The liver and spleen were weighed and ground separately in mortars in sterile physiological saline. Dilutions of the suspension were made and plated on GCBA. At 14 days after injection, the intraperitoneally injected mice and equal numbers of normal mice were injected intravenously with the fully virulent parent Sm strain. The mice were sacrificed, and the number of organisms in various tissues was determined as above.

The results are given in tables 1 and 2. The total number of organisms per mouse was calculated on the assumption that, on the average, each mouse contains 1.1 ml of blood and that the blood, liver, and spleen contain the majority of the organisms, although it is recognized that other parenchymatous organs also may serve as centers of multiplication. Earlier studies have shown that the kidneys and adrenal glands are involved, as well as the lymphoid tissue.

The number of organisms recovered at 4, 24,

TABLE 1

The multiplication of avirulent variants of Pasteurella tularensis in normal mice

Variant	Number of Organisms ($\times 10^3$) Recovered from Mice at Indicated Hours after Intravenous Injection of Approximately 1,200,000 Cells															
	Heart blood (per ml)				Spleen (per g)				Liver (per g)				Total per mouse			
	4 hr	24 hr	48 hr	96 hr	4 hr	24 hr	48 hr	96 hr	4 hr	24 hr	48 hr	96 hr	4 hr	24 hr	48 hr	96 hr
Jap_4	100	1.2	1.6	.26	5000	12,000	16,000	500,000	310	1600	1000	79				
Jap_6	310	1.2	.79	.026	7900	2,000	1,000	79	64	31	310	31				
	(per tissue)				(per tissue)				(per tissue)							
Jap_4	420	2.3	3.1	0.5	1600	2,600	40,000	2,000	5500	500	1200	1000	7520	3102	41,203	3000
Jap_6	640	20	2.0	0.5	1600	310	640	1,000	790	40	640	40	3030	370	1,282	1040

48 and 96 hours from mice injected with Jap_4 and Jap_6 variants is given in table 1. Organisms rapidly disappeared from the blood stream following injection with either Jap_4 or Jap_6. Mice injected with Jap_4 maintained a much higher number of organisms per gram of liver and spleen than did those injected with Jap_6. Multiplication in the spleen was marked in mice injected with Jap_4 in contrast with a significant decline in the case of Jap_6. Increase in the numbers of Jap_4 was more marked per tissue and per mouse than that of Jap_6.

It may be postulated from these results that during the multiplication of Jap_4 in the liver and spleen in mice a protective antigen is produced which is not produced in the case of Jap_6, where multiplication is much less active or where the total amount of antigen produced is less.

Table 2 gives the results of quantitative culture of tissues from mice which had recovered from an infection with Jap_4 and Jap_6 and which had been challenged with 500,000 cells of a virulent strain.

The increase in organisms per gram of tissue was greater in the spleen than in the liver in each case. Multiplication of the challenge organism in the spleen at 48 hours and in the liver at 72 hours was decidedly more marked in Jap_6 and in normal mice than in Jap_4 mice. When the total number of organisms per mouse is considered, it may be seen that multiplication of the fully virulent challenge organisms was inhibited in

TABLE 2

The influence of recovery from infection with avirulent variants upon multiplication of fully virulent Sm A variant

Immunizing Variant	Number of Organisms ($\times 10^6$) Recovered from Mice at Indicated Hours Following Intravenous Challenge with Approximately 500,000 Cells of Sm A																Mortality after Challenge
	Heart blood (per ml)				Spleen (per gram)				Liver (per gram)				Total per mouse				
	0 hr	24 hr	48 hr	72 hr	0 hr	24 hr	48 hr	72 hr	0 hr	24 hr	48 hr	72 hr	0 hr	24 hr	48 hr	72 hr	100 LD_{50}
Jap_4	0.1	.03	.39	.22	.75	1,300	6,500	300	.72	300	650	100					20
Jap_6	.32	.01	.28	2.5	.45	1,300	15,000	10,000	1.8	190	880	10,000					100
Normal	C*	5.0	6.0	0.8	.12	3,500	27,000	6,400	.08	1,200	20,000	640					100
	(per tissue)				(per tissue)				(per tissue)								
Jap_4	2.4	.81	8.8	4.8	0.3	210	1,200	90	.81	420	970	14	3.5	639	2,179	109	
Jap_6	6.2	.47	4.9	45	.04	300	4,800	1,500	1.5	210	1,400	17,000	7.7	510	6,204	18,545	
Normal	C*	12	120	21	.02	630	5,700	1,200	.09	1,800	2,400	1,000	0.1	2,442	5,820	2,221	

* C = Contaminated.

immune (Jap_4 recovered) mice in contrast with the rapid proliferation permitted in nonimmune mice that had received prior treatment with Jap_6. Normal mice showed a greater number of organisms at 24 hours than the Jap_4 or Jap_6 mice, and, although the average number of organisms at 72 hours was lower than in the Jap_6 mice, it was higher than in Jap_4 mice. These results indicate that protection in mice is contingent upon the ability of the animal to suppress the establishment of a virulent organism and that prior experience with a nonimmunogenic strain such as Jap_6 results in only a slight and transient ability to limit the multiplication of a virulent strain.

The ability to overcome the infection is shown also by the mortality percentage as shown in table 2. Mice recovered from Jap_4 infection gave only 20 per cent mortality after challenge with 100 LD_{50} of the virulent strain, whereas mice which had recovered from Jap_6 gave 100 per cent mortality after challenge. The normal control mice also gave 100 per cent mortality.

Preliminary experiments indicated that several smooth Jap variants produced a greater agglutinin response in rabbits than a nonsmooth variant, but did not induce immunity to challenge. The smooth variants also multiplied more actively in mice and induced after recovery of the mice a greater degree of ability to suppress multiplication of challenge strain than the nonsmooth variant. Accordingly, the following experiments were designed to follow the antigenic and immunogenic response in naturally susceptible and naturally resistant animals to the injection of living smooth and nonsmooth strains of *P. tularensis*.

The antigenicity and immunogenicity of smooth and nonsmooth variants of P. tularensis in normal and recovered susceptible and resistant animals.

The highly susceptible animals used in the experiment were rabbits, guinea pigs, and white mice; the more resistant animals tested were chickens and white rats. The following four variants were tested in each species: Ri_1, Ri_2, Jap_3, and Jap_5. Ri_1 and Jap_3 are smooth and immunogenic, whereas Ri_2 and Jap_5 are nonsmooth and nonimmunogenic in mice.

A series of three injections of living organisms was given on alternate days. This injection series was repeated 15 days after the first series. Sublethal numbers of organisms, depending upon the virulence of the variant, were injected per dose. The rabbits and rats were injected intravenously, while the guinea pigs, mice, and chickens were injected intraperitoneally. Five rabbits, guinea pigs, chickens, and rats were tested with each variant. Enough mice were injected so that 3 could be sacrificed at each bleeding time and 12 could be challenged at the proper time.

Agglutination titers on the individual animals were determined at 2, 4, 6, 10, 21, 27, 29, 31, and 35 days after the initial injection. Titers for mice were determined on pooled serum obtained from three mice at each bleeding. The results of those tests made through the 10th day are to be considered the result of injecting normal animals and the remainder the result of injecting recovered animals. Formalinized antigen prepared from the 38 strain was used in determining the agglutinin titer for each of the sera.

On the 14th day after the final injection each surviving animal was challenged intraperitoneally with the dilutions of a suspension containing approximately 2 to 4 billion organisms of Sm A, as indicated in table 1. Five normal animals were injected with a similar dose. In the case of mice, 12 recovered and 12 normal animals were challenged. The day of death of the animals is also shown in the table.

The results of the antigenic determinations are plotted in figures 1 and 2. An average of the titers acquired by the five animals tested, or three in the case of mice, is represented. The reciprocal values are shown. In figure 1 the results are arranged to compare the response of the various species to a given variant as follows:

Antigenicity of smooth Ri_1. It is indicated that susceptible normal rabbits respond immediately to a high level following the first injection series only; guinea pigs respond more slowly but to a high level; the response of the mouse is much slower and does not attain agglutinin titers as high as those of rabbits and guinea pigs. In recovered animals a sharp rise in titer occurs in guinea pigs, but little change occurs in rabbits and mice as a result of a second injection series.

The response of the normal resistant rat is immediate and very rapidly reaches a high titer; chickens, however, react more slowly and attain a much lower titer. As a result of a second injection series, both recovered rats and chickens respond with an immediate rise in titer, which levels off to a similar titer for each species by the 35th day.

Antigenicity of nonsmooth Ri_2. The over-all

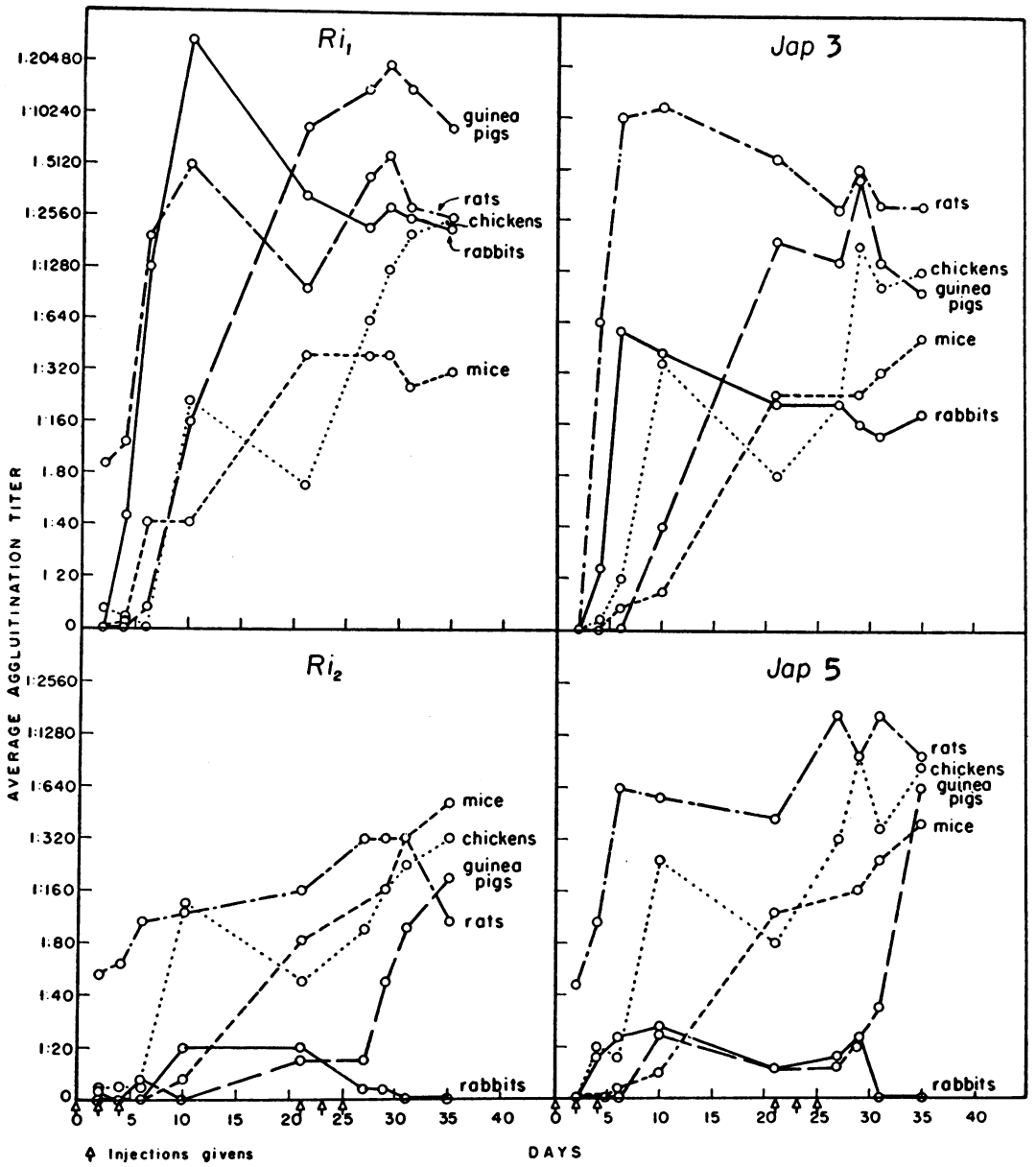

Figure 1. A comparison of the antigenic response among five animal species to the indicated variants of *Pasteurella tularensis*.

response is retarded in comparison with that induced by Ri_1. No significant titer was reached in normal rabbits and guinea pigs after the first injection series; the response was slightly better in mice. Following the second injection series the titer in guinea pigs and mice was increased; in rabbits the titer decreased.

Antibodies were detected earlier and to a higher level in resistant rats and chickens than in susceptible animals. After the second injection series recovered rats and chickens reacted with a sharp rise in titer.

Antigenicity of smooth Jap_3. An immediate rise in titer resulted in rabbits, whereas guinea pigs and mice responded more slowly. Guinea pigs eventually attained an even higher titer than did rabbits. As in the case of Ri_1, the titer in recovered rabbits remained unchanged, or de-

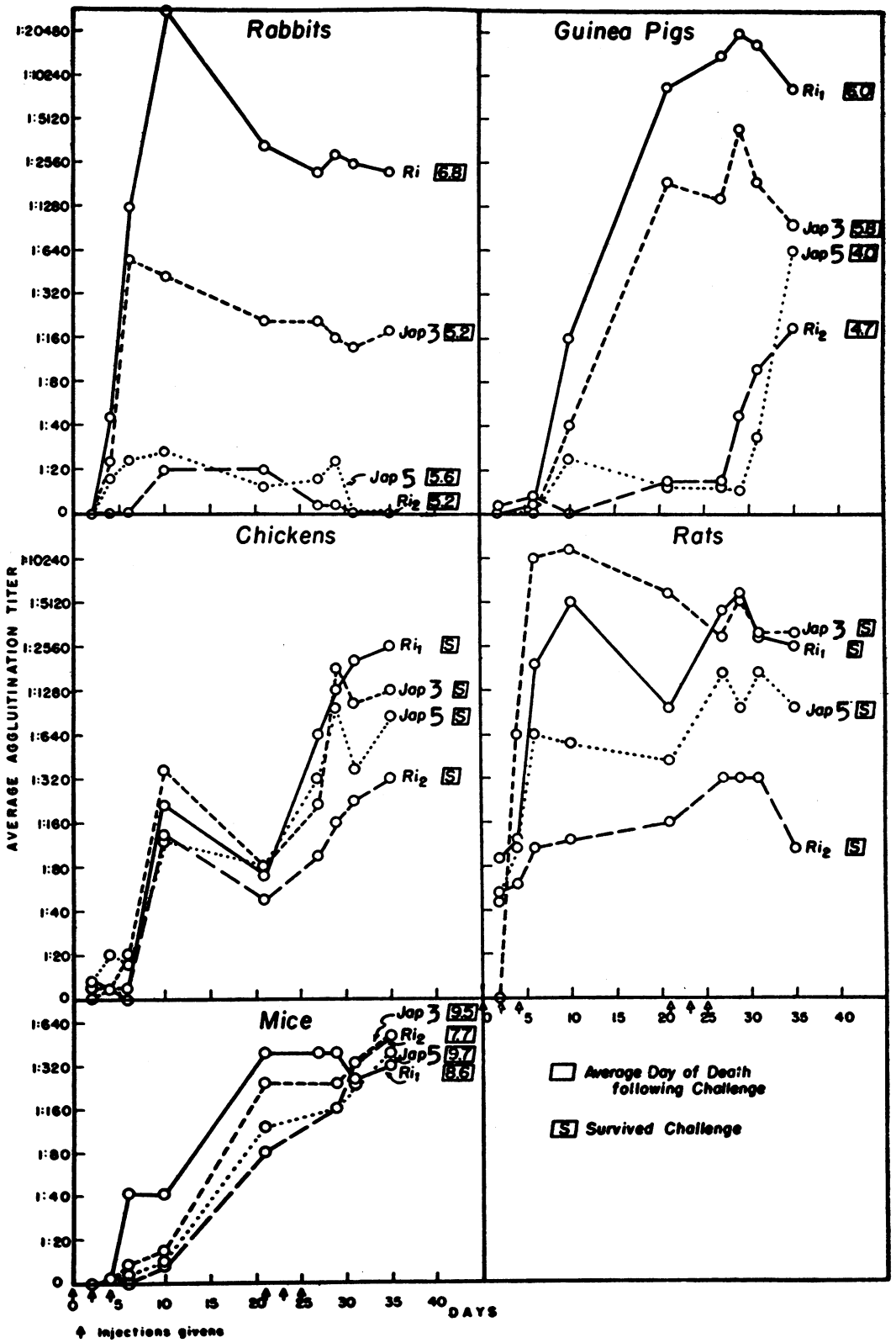

Figure 2. A comparison of the antigenic response induced by four variants of *Pasteurella tularensis* in the five animal species indicated.

creased, following a second series of injections. A definite rise occurred in guinea pigs, but only a meager response resulted in mice.

A very high titer was quickly reached in normal rats; the titer in normal chickens approximated that of rabbits. Following a second series of injections, the titer in rats changed very little but rose decidedly in chickens. The response in chickens was similar to that produced by Ri_1.

Antigenicity of nonsmooth Jap$_5$. The antigenic picture resulting from the injection of this variant was similar to that produced by the nonsmooth Ri_2 variant in each species of animal. The titer of rats and chickens, however, reached a higher level following the second injection series.

In figure 2 the results are arranged to compare the antigenic response of the variants in a given animal species as follows:

The antigenic response of rabbits. In normal rabbits both smooth variants, Ri_1 and Jap$_3$, caused a rapid rise in titer; the response to the nonsmooth variants, Ri_2 and Jap$_5$, was insignificant throughout the experiment. The change in titer occurring in recovered rabbits injected with Ri_1 and Jap$_3$ was not significant. In other words, the anamnestic response did not occur. Rabbits injected with Ri_1 maintained a much higher titer than did any of the other animals during the experiment.

The antigenic response of guinea pigs. The titer following the initial injections of smooth Ri_1 and Jap$_3$ into guinea pigs rose more slowly than in rabbits; however a second injection series evoked an initial rise which was followed by a slight drop. No significant titer developed in guinea pigs injected with nonsmooth Ri_2 and Jap$_5$ until after the second injection series, after which it rose sharply but to a limited degree. Again, as in rabbits, guinea pigs injected with Ri_1 maintained the highest titer during the experiment.

The antigenic response of mice. The response was similar for all four variants. A second series of injections of all variants except Ri_1 evoked a slight rise in titer. The antigenic pattern for mice shows a gradual rise in titer for all variants. The titer is high for mice but low in comparison with that induced in the other animal species tested.

The antigenic response of chickens. Chickens responded in the same manner to the four variants, being the least responsive to Ri_2. In each case a similar agglutinin titer resulted from each variant within 10 days following the injection of normal animals. In recovered animals a very marked rise in titer occurred and reached a much higher level than when normal animals were injected.

The antigenic response of rats. The injection of smooth Jap$_3$ caused an immediate rise to a very high titer in normal rats which was followed by a decline. The response to Ri_1 was also very pronounced. A weaker response occurred in the case of the nonsmooth Jap$_5$ and Ri_2 variants. Ri_1, Jap$_3$, and Ri_2 caused an anamnestic response in recovered rats.

A comparison of titer at the time of challenge with the degree of protection is indicated in table 2 and in figure 2. As shown by the average day of death of normal animals injected with approximately 100 organisms of Sm A, rabbits, guinea pigs, and mice were highly susceptible. Normal chickens were resistant to at least 10 million organisms of Sm A. When recovered rabbits were challenged, the average day of death was 1.6 to 3.2 days later than in normal animals injected with a similar dose. No complete protection of the rabbits resulted despite the presence of a high agglutinin titer. Recovery from Ri_1 injections resulted in the longest survival following challenge. In recovered guinea pigs the average day of death following challenge was 1.0 to 3.0 days later than that in normal animals. Again, Ri_1 afforded the greatest protection, though not complete. Seventy-five per cent of the mice recovered from Ri_1 injections survived a challenge of 100 LD_{50} doses of Sm A. Jap$_3$ protected 50 per cent; Ri_2, 8 per cent; and Jap$_5$, 25 per cent of the mice challenged with 100 LD_{50} doses of Sm A. The average day of death in all recovered mice was delayed at least 4 days when compared with normal mice.

Challenge of the naturally resistant animals showed that all normal and recovered chickens survived an injection of approximately 100 million organisms, only 1 to 4 of which were required to kill a mouse. All recovered rats, except one, survived a challenge dose of 10 million Sm A organisms. All normal rats died by the second day. The short interval of survival after challenge of the normal rats indicates a high degree of protection in the recovered rats, following infection with each of the four variants.

There was no apparent consistent correlation

between agglutinin titer and protection against challenge. For example, some rabbits having no agglutinin titer survived as long as certain rabbits having a high titer. Also, chickens and rats having either very high or very low titers survived the challenge dose.

This experiment demonstrates again the superior antigenic potency of smooth, more virulent variants of *P. tularensis* over the nonsmooth, less virulent variants. It also illustrates the differences in host response. The pattern of the antigenic response cannot be correlated completely with the degree of susceptibility of the animals to tularemia. Antigenic differences among variants appear to be most obvious when tested in guinea pigs and rabbits. It was interesting to note that the susceptible guinea pigs and rabbits responded markedly only to the smooth, more virulent variants, whereas the resistant chicken and rat responded reasonably well to both smooth and nonsmooth variants. Highly susceptible mice also responded almost equally well to each type of variant though not to levels as high as those attained in resistant species. With the exception of rabbits, both susceptible and resistant animals responded to the second injection series of both smooth and nonsmooth variants with a slight rise in titer which soon dropped slightly. In spite of the large number of living organisms injected into rabbits and guinea pigs and their recovery from these injections, insufficient immune response was elicited to protect them from 100 mouse LD_{50} of virulent organisms. Previous work by Pannell and Downs (1953) has shown that circulating antibodies play only a slight protective role in experimental tularemia. The ability of immunized rats to dispose of virulent organisms seems to be the basis for their resistance to virulent organisms as shown by Downs *et al.* (1949).

DISCUSSION

The importance of the host species, as well as the behavior of the bacterial variants, is illustrated in the experiments reported here. The results of quantitative culture after the injection of these variants seem to indicate that the immunogenicity of Jap_4 is dependent on its ability to multiply in the mouse. Since both of these strains are smooth, the component responsible for this character is not identical with the immunogenic component. It might be suggested that, in association with this ability of immunogenic variants to multiply, there is an antigenic factor not possessed by nonimmunogenic variants but as yet not demonstrated. The necessity for multiplication of the invading organism in tularemia infection with strains of low virulence in the production of immunity appears to be similar to that in plague, as reported by Walker *et al.* (1953). They found that avirulent strains which were effective as immunogenic agents although incapable of extensive multiplication, were either rich in envelope antigen or had the capacity to multiply enough to produce the necessary amount of antigen.

Dubos *et al.* (1953) have recently shown that attenuated tubercle bacilli which convey immunity are those which multiply in the host to an appreciable degree, and that avirulent bacilli which do not multiply in the host confer no immunity to challenge with virulent organisms.

The experiments in which two resistant hosts, rats and chickens, and three susceptible hosts, mice, guinea pigs, and rabbits, were used show that, in both resistant and susceptible hosts, infection with smooth strains results in a greater production of agglutinating antibodies than infection with nonsmooth strains. Whether the organisms were able to multiply in the hosts seemed not to affect the outcome of challenge, since all of the animals received very large but sublethal numbers of living organisms over a period of 25 days on two separate occasions. Quantitative studies had shown that Ri_1 and Jap_3 multiplied well in the mouse and were immunogenic, whereas Ri_2 and Jap_5 multiplied poorly and were not immunogenic. In this host, however, all strains were antigenic to approximately the same degree, although Ri_1 and Jap_3, the smooth strains, induced a faster rise in antibodies than did the nonsmooth strains. In rabbits and guinea pigs, in spite of an active production of agglutinins against the smooth strains and the presence of agglutinins in relatively high titer at the time of challenge with 10 or 100 LD_{50} of a virulent strain, these animals succumbed without any evidence of resistance, except for a slight delay in time of death as compared with the controls. The rabbits infected prior to challenge with the nonsmooth strains showed no differences in resistance from those infected with the smooth strains, although they did develop a higher titer of agglutinins to the smooth strains. Rats and

chickens showed somewhat better agglutinin production after infection with smooth strains. The animals infected with the nonsmooth strains were just as resistant to challenge with a virulent strain as those receiving the smooth strains. The marked resistance of the rat and chicken may depend upon a very rapid response to the injection of the living organism and to the ability to dispose of it rapidly. This rapid response is suggested by the quantitative studies in rats by Downs *et al.* (1949) in which they showed that vaccinated and recovered rats rapidly limited multiplication of the challenge organisms and disposed of them, although invasion of the spleen and liver took place by way of the blood stream.

It is evident from the results presented here that the behavior of variants must be defined in terms of the host, whether susceptible or resistant, and whether capable of responding to the immunogenic stimulus.

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SUMMARY

It has been demonstrated that smooth variants which multiply in the white mouse are immunogenic, whereas smooth variants which do not multiply are not immunogenic. The possibility of the existence of an antigen, as yet undemonstrated, corresponding with the ability of the strain to multiply and to immunize is considered.

When living smooth variants were injected, they were more capable of stimulating a higher titer of agglutinins in both susceptible and resistant hosts than nonsmooth variants.

Smooth variants which were immunogenic in white mice were not immunogenic in rabbits or guinea pigs.

Both smooth and nonsmooth variants were immunogenic in rats and chickens.

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