

ANTITULARENSE SERUM: CORRELATION BETWEEN PROTECTIVE CAPACITY FOR WHITE RATS AND PRECIPITABLE ANTIBODY CONTENT

By L. FOSHAY, I. RUCHMAN AND P. S. NICHOLS

(From the Department of Bacteriology, College of Medicine, University of Cincinnati, and the Cincinnati General Hospital)

(Received for publication July 21, 1946)

Heretofore all attempts to demonstrate protective antibody in antitularense serums have failed whenever animals were challenged with a strain of high virulence. Previous test animals have been the mouse, guinea pig, hamster, and rabbit. Sources of immune or hyperimmune serums were goats, horses, sheep, rabbits, and man. Our considerable unpublished experience with these animals and serums is in good agreement with the reports of Francis and Felton (1) and Bell and Kahn (2). If injected with serum before challenge these animals will usually exhibit significant prolongations of the survival time beyond that of control animals, but no actual survivals against as little as 1 to 10 M.L.D. of a virulent challenge strain.

The inability to demonstrate serum protection in these highly susceptible animals has contributed to a widespread but unfounded belief that immune and hyperimmune serums are ineffective therapeutic agents in human tularemia. Although reports by Foshay (3, 4) on the results of serum therapy in individual patients, as well as analyses of accumulated data derived from the independent observations of more than 600 clinical observers, have continued to demonstrate significant reductions in mortality and highly significant reductions in morbidity in comparison with comparable data from untreated patients, universal acceptance of the value of serum therapy has been impeded by a lack of suitable means to demonstrate protective antibody and to establish quantitative criteria for potency. The work here reported was undertaken to supply these deficiencies.

The inability of the highly susceptible mouse, guinea pig, hamster, and rabbit to react with any useful degree of resistance before death occurs following infection with a single virulent unit of *Bacterium tularense* makes these animals unsuitable test hosts for serum protection experiments.

The reasons for this inability, obviously inoperative for many other infectious agents under similar conditions of testing, are unknown at present. The reactions of these animals to invasion are quite unlike that of man, for whom there is ample evidence that the great majority rapidly develop a high degree of resistance, resulting in a disease that is characterized clinically by a low mortality and, pathologically, by tissue reactions that soon exhibit subacuteness and chronicity.

During the course of the initial investigations on this disease, McCoy (5) noted that rats, both gray and white, were highly resistant to cutaneous infection and considerably resistant to subcutaneous inoculation. Infection was proved by demonstration that the spleens of rapidly recovered rats harbored the virulent organisms. Since the reaction to invasion by the rat more closely approximates that of man than do those of other laboratory animals, the rat was chosen as the test animal for serum protection studies.

MATERIALS AND METHODS

Rats. All rats employed were of the Wistar strain. Special care was taken to secure groups of animals of similar weight in order to insure uniformity of results. The permissible variation in weight was from 70 to 115 grams, and the great majority varied between 85 and 110 grams.

Challenge strain. The strain of *Bacterium tularense* used was the highly virulent strain SCHU, which had been studied extensively and had been maintained frequently in animal passage since its isolation in 1941. Its subcutaneous LD₅₀ titers for mice, guinea pigs, and rabbits varied from 9.1 to 9.7/0.5 ml. The LD₅₀ titers were determined by the method of Reed and Muench (6). They are expressed as the logarithms of the dilutions, disregarding the minus sign.

Challenge suspensions. Challenge suspensions were prepared from the second of 2 consecutive 16-hour subcultures on glucose glycerol cystine human blood agar. The growth was placed in physiological salt solution and suspended evenly by means of a fine bore pipette. Each

suspension was adjusted to a turbidity of 40 microamperes (M.A. 40) in 18-mm. tubes by means of a reflecting photoelectric comparator. These 10^8 suspensions were used to make serial decimal dilutions in physiological salt solution.

Challenge dose. The challenge dose for all rats was 1 ml. of M.A. 40×10^2 dilution, administered subcutaneously. Many plate counts made by the method of Downs, Coriell, Chapman, and Klauber, as well as numerous virulence titrations in mice, showed that the M.A. 40 suspension contained from 2.5 to 3 billions of viable organisms per ml. Hence, the challenge doses contained at least 25 millions of viable, virulent organisms.

Serums. The serums used had the following histories.

A. Hyperimmune goat serum, pooled from bleedings of 4 bucks that had been inoculated intravenously 3 times a week for 5 months with saline suspensions of living strain SCHU. The initial agglutinin titer was 1:20,480; at the time of rat testing it was 1:5,120. The unconcentrated serum, preserved with merthiolate to 1:30,000, had been stored in 60-ml. quantities at 5° C. This serum was 15 months old at the time of the first protection test.

B. Hyperimmune goat serum, pooled from bleedings of 3 bucks that had been inoculated subcutaneously 3 times a week for 1 month with saline suspensions of living strain SCHU. Each of these animals had been inoculated intravenously during the previous year, for preparation of serum A. The initial agglutinin titer was 1:10,240+; at the time of rat testing it was 1:1,280. Unconcentrated serum was preserved with merthiolate to 1:30,000 and held in 60-ml. quantities at 5° C. This serum was 4 months old at the time of the first test.

C. Hyperimmune horse serum No. 50737, obtained from Sharp & Dohme; pooled from 8 bleedings of 1 horse. This animal had been inoculated intravenously twice a week with formalin-killed suspensions of strain SCHU for 11½ months during 1942. During 1943 it was inoculated intravenously 3 times a week for 2 months with suspensions of living strain SCHU and, similarly, with living suspensions for 4 months during the next year. Thereafter, in order to avoid infection of unprotected personnel, formalin-killed suspensions of this strain were injected intravenously as maintenance doses during the first half of the bleeding period. During the second half of this period maintenance inoculations consisted of intravenous injections of phenol-killed whole cultures of strain SCHU in a semisynthetic soybean hydrolyzate liquid medium. Both unwashed killed suspensions contained about 2.5 billions of bacteria per ml., and included the minute minimal reproductive units of the organism. The weekly dosage was 2 ml. At the time this unconcentrated serum was tested its pooled bleeding aliquots varied in age from 14 to 6 months, and the agglutinin titer was 1:1,280.

D. Aged immune goat serum No. 79409, prepared in 1940-1941 by Sharp & Dohme by intravenous inoculations for 8 months of unwashed heat-killed suspensions of strains of maximal virulence. Pooled serum from 6 goats was preserved with phenol and refrigerated in

liquid bulk. At the time of testing it was more than 5 years old, and its agglutinin titer was 1:1,280.

E. Normal horse serum No. 51975, without preservative, obtained from Sharp & Dohme.

F. Normal goat serum prepared from 2 bleedings of an uninoculated kid born of uninoculated parents. One lot contained merthiolate to 1:30,000; the other contained no preservative.

Quantitation of precipitable antibody. The precipitable antibody content of serums was determined by the neutralization method of Culbertson (7). The antigen was a nonimmunogenic polysaccharide prepared from acetone extracted cells of strain SCHU by the phenol extraction method of Palmer and Gerlough (8). Antibody content was usually measured with antigen in steps of 10 micrograms. Serum of low antibody content and dilutions of serums of high antibody content were quantitated with sharp end points with antigen increments in steps of 1 microgram. Antibody content is expressed as mgm. of antigen necessary to exhaust antibody from 1 ml. of serum without resultant antigen excess.

Serum protection tests. Serum protection tests were performed in the following manner. Rats were inoculated in groups of 30 to 32, half of each group receiving one of the potent serums and the other half receiving either the corresponding normal serum or the aged immune goat serum. Each rat was injected intraperitoneally with 2 ml. of undiluted serum and immediately thereafter with the 1-ml. challenge dose, subcutaneously, into the abdominal wall near the groin. The 2-ml. serum dosage was selected with reference to the agglutinin titer curves of pooled serums from normal rats after intraperitoneal injections of an immune goat serum with a titer of 1:5,120. Rats that received 2 ml. of serum had titers of 1:1,280 at 24 hours, and 1:160 at 48 hours. Rats that received 1 ml. of serum had titers of 1:160 at 24 hours, and zero in 1:10 dilution at 48 hours. Deaths were recorded daily for 21 days before release. The infecting strain was recovered from a rat dying on the second or third day from each group, and was used to infect each succeeding group.

Determination of persistence of infection in the spleens of recovered rats. After release all recovered animals were held for sacrifice at weekly intervals from 3 weeks to 3 months after challenge and were tested for residual splenic infection by cultures and by intraperitoneal inoculations into mice of suspensions of ground spleens.

EXPERIMENTAL RESULTS

Preliminary virulence titrations in normal rats. Before undertaking protection experiments the optimal subcutaneous challenge dose was determined by virulence titrations in normal rats weighing 70 to 135 grams. Preliminary subcutaneous titrations in adult rats of 200 to 220 grams showed great irregularity of mortality in respect to challenge doses. Mortality was frequently 100 per

cent from M.A. 40×10^{-1} , 10^{-3} , 10^{-4} , and 10^{-9} dilutions, and only 20 to 60 per cent from the intervening dilutions. An increase in the number of rats used per dilution reduced but did not dispel this irregularity. Although rats are highly susceptible to intraperitoneal challenge, yielding LD_{50} titers/1 ml. equivalent to those obtainable in mice injected either S.C. or I.P./0.5 ml., there is great variation in natural resistance among individual rats that are challenged subcutaneously, and this variable resistance apparently increases with age. Subcutaneous titrations in weight groups of 75 to 95 grams and of 95 to 115 grams revealed less irregularity due to variable individual resistance and gave separate LD_{50} titers of 6.4.

The cumulative mortalities and LD_{50} titers from 6 titrations in rats weighing from 70 to 135 grams are shown in Table I. The LD_{50} titers for the separate titrations were 7.6, 8.4, 5.6, 5.2, 8.0, and 6.0, and the cumulative LD_{50} titer was 6.4. This titer became stabilized between 6.3 and 6.5 only after 90 rats were used. The cumulative intraperitoneal mortalities and LD_{50} titers for rats weighing 70 to 210 grams, with separate titers of 9.3, 8.6, 9.5, 9.7, and 8.4, were obtained from simultaneous titrations with the same dilutions used for the subcutaneous titrations. The cumulative intraperitoneal titer of 9.3 indicates that the rat is about 1,000-fold more resistant to subcutaneous challenge than to intraperitoneal challenge. The relatively minor differences among the intraperitoneal LD_{50} titers indicate that the greater variability observed among subcutaneous LD_{50} titers was caused by differences in individual resistance among rats, and was not owing to lack of uniformity of the separate M.A. 40 suspensions

or to errors in decimal dilutions prepared from them. The comparison between subcutaneous and intraperitoneal virulence titrations in mice, many performed simultaneously with the same dilutions, shows that the mouse does not exhibit differences in resistance dependent upon the route of challenge and that the total lack of resistance to experimental infection was shared by all mice.

Since 2 consecutive subcutaneous virulence titrations in the variably resistant rat, 30 and 39 animals being used, with simultaneous intraperitoneal titrations with 9 rats each, gave subcutaneous LD_{50} titers of 8.0 and 5.2, and intraperitoneal titers of 8.4 and 9.7, respectively, it became apparent that virulence titrations, if performed simultaneously with serum protection tests, and with fewer than 90 rats per titration, would not permit calculation of the actual challenge doses received by the protected rats. Calculations made from the separate subcutaneous titrations showed that the challenge dose used in protection experiments might have varied from 1,600 to 250,000 LD_{50} doses. Since it was not feasible to use 90 animals for each titration and protection series, and since the M.A. 40×10^{-2} dilution killed regularly at least 96 per cent of rats of the greater weight range, and at the same time permitted protection of more than 50 per cent of animals in preliminary protection tests, the 10^{-2} dilution was adopted for challenge of all rats of the narrower weight range in the protection experiments. For similar reasons it seemed advisable to express the challenge dose as an average in terms of the cumulative subcutaneous LD_{50} titer. On this basis each rat in the following protection experiments received 25,000 LD_{50} doses.

TABLE I

Virulence titrations with strain SCHU in 70 to 135 grams white rats and in 18 to 25 grams white mice

Comparative mortalities and cumulative LD_{50} titers after subcutaneous and intraperitoneal inoculation. Dosage was 1 ml. for rats, 0.5 ml. for mice.

Animal	Route	Decimal dilutions of strain SCHU from M.A. 40 suspension										Cumulative LD_{50} titers
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
Rat	S.C.	9/9*	30/31	17/20	21/26	15/26	13/24	12/24	10/24	4/21	1/18	6.4
	I.P.							8/8	15/16	11/17	2/14	9.3
Mouse	S.C.							14/14	15/15	10/15	1/15	9.3
	I.P.							25/25	34/34	26/35	9/35	9.4

* 9/9 = 9 of 9 animals injected died of tularemia.

Serum protection of rats in relation to precipitable antibody content. The results of serum protection experiments are given in Table II. In-

at 4 weeks. Thereafter, weekly tests yielded the organisms only twice, from single rats, at the eighth and thirteenth weeks.

TABLE II

Correlation between precipitable antibody content of antitularense serums and protective capacity for white rats

No. of rats	Group	Serums	Maximal agglit. titers	Mor-tality	Sur-vival	Anti-body content
					per cent	mgm. per ml.
16 16	A	Hyperimmune goat by I.V. inoc.	1:5120	2/16 2/16	87.5	1.10
16 15	B	Hyperimmune goat by S.C. inoc.	1:1280	6/16 2/15	74.2	0.7
16 16	C	Hyperimmune horse by I.V. inoc. no. 50737	1:1280	7/16 3/16	68.7	0.5
15 16	D	Aged immune goat by I.V. inoc. no. 79409	1:1280	9/15 6/16	51.6	0.085
15 15	E	Normal horse no. 51975	1:10	15/15 13/15	6.6	0.001
15 15	F	Normal goat	0	14/15 15/15	3.3	0

stead of comparing each potent serum with its respective control serum all serums are tabulated in descending order with respect to precipitable antibody content. Each serum was tested on 2 separate occasions, and the number of animals and the mortality ratios are shown for each test in order to indicate the residual effect of variable resistance among rats within a weight range of 85 to 110 grams. These differences are negligible for the most potent serum and for both normal serums. The results showed good correlation between survival percentages and mgm. equivalents of antibody content but little correlation between antibody content and agglutinin titers. The low agglutinin titer and the low precipitable antibody content of the normal horse serum were observed independently and were verified repeatedly, but we have no explanation for their presence.

Isolation of the infecting strain from recovered rats. Mouse inoculation proved to be more reliable than cultures for the recovery of organisms from suspensions of ground spleens. Infected spleens were demonstrated regularly at 3 weeks after challenge, and with a frequency of 75 per cent

DISCUSSION

The decisive results of the serum-protection experiments demonstrated conclusively that protective antibody was present in antitularense serums. The observed differences in mortality between rats that received immune serums and those that received normal serums are significant by inspection. The degrees of significance of the mortality rates from groups A, B, C and D, in comparison with the rate obtained after pretreatment with normal horse serum, are extremely high, all values of *p* being so small that they are unimaginable figures. Since the virulence titrations in rats having a slightly higher maximal weight limit showed that stability was not reliably secured with fewer than 90 rats, we attach less significance to the observed differences in mortality among groups A, B, C and D. Inspection of the paired mortality ratios for groups B, C and D reveals that variable individual resistance among rats in the narrower weight range was still operative. Hence, it is unlikely that significant differences in potency between these serums could be determined without using a much larger number of rats for each serum. Analysis of the rates for groups A, B and C, in comparison with the rate from group D, showed that no difference was significant, though that between groups D and A barely escaped statistical significance.

The protection test employed is satisfactory to determine the presence or absence of protective antibody. In the above tests it actually gave good agreement between rat survival and antibody content for all serums, and it is perhaps possible that repetition might continue to reveal proportional differences in protective antibody in serums of graded potency in agreement with their respective precipitable antibody contents, but, unless larger numbers of animals were used for each serum, conclusions about the relative potencies are not really justified. It is apparent from the mortality of rats in group D that a very small amount of antibody is sufficient to tip the balance heavily in favor of survival, even against a large challenge dose of a strain of maximal virulence.

The variable resistance of rats to subcutaneous challenge would have necessitated the use of so many animals per serum dilution that determinations of the ED₅₀ doses of serums were impractical. The possibility of securing more significant degrees of relative serum protection, with the same or a smaller number of animals, by means of a suitable intraperitoneal challenge dose has not been explored.

Although the protection test described provides a method for the demonstration of protective antibody in antitularemia serums it does not in its present form furnish an accurate basis for the establishment of quantitative criteria for potency. Further study of the precipitable antibody content in relation to protective capacity may eventually provide a serologic method to quantitate the potency of serums, thus obviating the use of test animals. Until a satisfactory method is devised temporary safeguards might be adopted. For example, a provisional standard might require a minimum of 1 mgm. equivalent of antibody per ml. of serum or perhaps a survival rate of 75 per cent among rats selected and challenged in accordance with the above specifications.

SUMMARY

Protective antibody was demonstrated in antitularemia serums against subcutaneous challenge with an average of 25,000 LD₅₀ doses of a strain of *Bacterium tularensis* of maximal virulence, using white rats of 85 to 110 grams as the test animals. Good correlation was observed between

protective antibody and precipitable antibody content, but not between antibody content and agglutinin titers.

ACKNOWLEDGMENT

We are grateful to our associates, Dr. W. B. Hesselbrock and Dr. I. W. Gibby, for assistance in the preparation of immune serums.

BIBLIOGRAPHY

1. Francis, E., and Felton, L. D., Antitularemia serum. Pub. Health Rep., 1942, 57, 44.
2. Bell, J. F., and Kahn, O. B., Efficacy of some drugs and biologic preparations as therapeutic agents for tularemia. Arch. Int. Med., 1945, 75, 155.
3. Foshay, Lee, Tularemia: A summary of certain aspects of the disease including methods for early diagnosis and the results of serum treatment in 600 patients. Medicine, 1940, 19, 1.
4. Foshay, Lee, A comparative study of the treatment of tularemia with immune serum, hyperimmune serum, and streptomycin. Am. J. Med., 1946, 1, 180.
5. McCoy, G. W., A plague-like disease of rodents. Pub. Health Bull. No. 43. Washington, April, 1911, pp. 53-71.
6. Reed, L. J., and Muench, H., A simple method of estimating fifty per cent endpoints. Am. J. Hyg., 1938, 27, 493.
7. Culbertson, J. T., A quantitative study of the precipitin reaction with special reference to crystalline egg albumin and its antibody. J. Immunol., 1932, 23, 439.
8. Palmer, J. W., and Gerlough, T. D., A simple method for preparing antigenic substances from the typhoid bacillus. Science, 1940, 92, 155.
9. Downs, C. M., Coriell, L. L., Chapman, S. S., and Klauber, A., The cultivation of *Bacterium tularensis* in embryonated eggs. J. Bact., 1947, 53, 89.