A QUANTITATIVE ANTIBODY RESPONSE OF MAN TO INFECTION OR VACCINATION WITH PASTEURELLA TULARENSIS*

By MARY M. ALEXANDER,‡ Ph.D.

(From the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland, and the Department of Bacteriology, Hygiene, and Preventive Medicine, the George Washington University, Washington, D. C.)

(Received for publication, April 10, 1950)

It has been demonstrated clinically in a large number of human subjects that vaccination against tularemia is of value. Foshay (1) has administered vaccines which demonstrably lowered the incidence of disease and lessened the severity of the symptoms in exposed groups. The therapeutic use of immune serum in human infections was also shown by Foshay (2) to be advantageous.

Experimentally, the value of either active or passive immunization has been difficult to demonstrate. Downs (3) and Larson (4) demonstrated some protection of rabbits and of white rats, respectively, following active immunization. Francis and Felton (5) were unable to obtain any evidence of protective antibody in sera from a variety of experimentally immunized animals. Larson (6) showed evidence of protective antibody in sera from recovered and vaccinated human beings. Recently Foshay *et al.* (7) found a correlation between the protective titre for rats in sera from immune goats and horses, and the precipitable antibody when a polysaccharide extracted from *Pasteurella tularensis* was used as the test antigen. The present study is concerned with the immune response of the vaccinated or recovered person to tularemia, as indicated by a measurement of antibody present in the serum which is reactive with a similar polysaccharide antigen.

Materials and Methods

Sera.—Human sera included sixteen samples from individuals who had recovered from tularemia; eleven from individuals who had received Foshay's vaccine; two from individuals previously recovered from the disease who suffered reinfections; and three from individuals who had had no known contact with the organism. Unless otherwise stated, vaccinated persons were bled 2 weeks following the last injection of one or more series.

The blood was allowed to clot at room temperature and was stored in the refrigerator overnight before removal of the serum. Unless otherwise specified, complement was removed by the addition of egg albumin containing 0.01 mg. N, and rabbit anti-egg albumin contain-

* From a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The George Washington University, Washington, D. C.

‡ Present address: Roscoe B. Jackson Memorial Laboratory, Bar Harbor.

ing 0.1 mg. N, per ml. of serum, followed by incubation for 3 hours at 37°C. and 48 hours at 0–4°C. The resulting precipitate was removed by centrifugation.

Antigen.—The antigen used was a polysaccharide fraction of *Past. tularensis*, strain Schu, which was isolated by the method of Palmer and Gerlough (8) in the manner described by Nicholes (9). Organisms were grown in Snyder's liquid peptone medium (10), each flask being inoculated with one-fifth volume of an 18 hour culture and incubated on a mechanical shaker for 24 hours at 37°C. The organisms were harvested by centrifugation at 0–4°C., washed twice with 0.85 per cent NaCl solution, extracted at 37°C. for 24 hours with acetone, dried *in vacuo* over $CaCl_2$, ground to a fine powder, and weighed. In two instances, 3.76 and 8.69 gm. of dried organisms were obtained from 15 liters of medium.

The organisms were extracted with liquefied crystalline phenol at 40°C. in a water bath. Five volumes of distilled water was added to the phenol-soluble fraction. The mixture was dialyzed in cellophane casing against running tap water, and then concentrated at 0–4°C. to approximately one-fourth volume by allowing a fan to blow on it. The concentrated fluid was centrifuged for 2 hours at 2,000 R.P.M. in an International No. 2 centrifuge; the supernatant fluid was then centrifuged for 15 minutes at 5,000 R.P.M. The final supernatant fluid was cooled to 0°C. and three volumes of cold 95 per cent ethyl alcohol was added while the flask was being shaken. The precipitate which formed was separated by centrifugation and redissolved in a small quantity of distilled water. Three reprecipitations were done, using alcohol which was made slightly alkaline to litmus with NaOH and which contained 0.2 per cent sodium acetate. The final precipitate was dissolved in distilled water and lyophilized. Concentrated solutions were made in 0.85 per cent NaCl solution and stored at −30°C.

Quantitative Precipitation.—The general technique of Heidelberger (11) was followed. Complement-free sera were added to a series of 15 ml. conical centrifuge tubes in duplicate or triplicate, in an amount dependent upon the probable amount of antibody. One or 2 ml. portions of sera were adequate when the serum was from a recovered person; a larger amount was necessary in sera from some vaccinated persons. In most cases the serum plus saline was added in a total quantity of 4 ml. To each tube containing a constant volume of diluted serum, 1 ml. of the variable concentrations of antigen was added; or in the case of controls, 1 ml. of 0.85 per cent NaCl solution. Concentrations of antigen were chosen to include three points in the antibody excess zone in which nearly 50 per cent of the total amount of antibody was expected to be precipitated. To insure sterility, aseptic technique was followed throughout, and merthiolate was added to a final concentration of 1–10,000. After thorough mixing, the tubes were incubated in a water bath at 37°C. for 3 hours, then at 0–4°C. for 10 to 14 days, during which time they were shaken every other day. The tubes were centrifuged at 0–4°C. and the precipitates washed three times with cold 0.85 per cent NaCl solution. The supernatant fluids were examined for the presence of excess antigen or antibody; the precipitates were analyzed for nitrogen using the colorimetric technique of Heidelberger and MacPherson (12).

The data were analyzed by the method of Heidelberger (11), including for each analysis only those points in the antibody excess zone in which 50 per cent or more of the total antibody was precipitated. The following values, all at the theoretical midpoint of the equivalence zone, were calculated for each serum: A, the amount of antibody N precipitated; G, the amount of antigen combined; and R, the ratio A/G.

EXPERIMENTAL RESULTS

The applicability of Heidelberger's equation to this antigen-antibody system, and its reliability in calculating the amount of nitrogen precipitable by any amount of antigen in the antibody excess zone are shown by the results re-

TABLE I

Comparison of Values of Precipitable Nitrogen Obtained in the Antibody Excess Zone Experimentally and by Heidelberger's Equation

G in precipitate	N precipitated (2 ml. serum)			Supernatant fluid
	Observed	Calculated 9 points	Calculated 2 points 0.10 and 0.15	
mg.	*mg.*	*mg.*	*mg.*	
0.05	0.025	0.025		Excess A
0.10	0.046	0.046	0.046	Excess A
0.12	0.053	0.053	0.053	Excess A
0.15	0.063	0.062	0.063	Excess A
0.17	0.069	0.068	0.068	Excess A
0.20	0.071	0.074	0.076	Excess A
0.22	0.077	0.078	0.079	Excess A
0.30	0.087	0.086	0.087	Faint trace A
0.32	0.087	0.087	0.087	Faint trace A

Calculated 9 points
mg. N = 0.541 G − 0.847 G^2
R = 0.271
A = 0.087

Calculated 2 points, 0.10 and 0.15
mg. N = 0.545 G − 0.85 G^2
R = 0.273
A = 0.087

TABLE II

Analyses of Normal Human Sera and of Sera from Human Beings Vaccinated with Past. tularensis

Serum No.*	No. series of vaccine‡	Serum used in analysis	Equivalence values (10 ml. serum)			Exposure after analysis
			A	R	G	
		ml.	*mg. N*		*mg.*	
VIII	5	8	0.041	0.18	0.23	Not exposed
XXXIV	5§	4	0.015			Not exposed
II	4	2	0.113	0.15	0.77	Exposed
IV	4	4	0.024	0.10	0.25	Not exposed
V	4	4	0.095	0.26	0.36	Not exposed
VII	4	8	<0.03			Not exposed
XXXIII	4‖	4	<0.006			Not exposed
VI	3	4	0.065	0.13	0.49	Not exposed
IX	3	4	0.045	0.11	0.42	Not exposed
XXIII	3	2	0.145	0.15	1.0	Exposed
III	2	2	0.113	0.11	1.08	Not exposed
XII	None	2	None			Not exposed
XIV	None	2	None			
XV	None	2	None			

* All sera were obtained 2 weeks following the last injection of a series unless otherwise specified.

‡ Each series composed of three injections of 0.5 ml. each of Foshay's vaccine, except the first, which was one injection of 0.25 ml. followed by three injections of 0.5 ml. If on any injection a systemic or skin reaction appeared, the series was considered complete without further injections in that series.

§ 3 years previously.

‖ 4 years previously.

corded in Table I. There is excellent agreement in the actual and theoretical values obtained, even when as few as two determinations in the antibody excess zone were used in calculating values.

TABLE III

Analyses of Sera of Human Beings Recovered from Tularemia

Serum No.*	Serum used	Time since infection	Equivalence values (10 ml. serum)			Exposure‡ after analysis
			A	R	G	
	ml.		*mg. N*		*mg.*	
XVII	1§	14 yrs.	0.259	0.31	0.84	Exposed
XVI	1§	10 yrs., 6 mos.	0.232	0.14	1.61	
XXI	2	7 yrs.	0.228	0.28	0.82	Exposed
X	2	4 yrs., 1 mo.	0.622	0.54	1.16	Exposed
X-a	2	2 wks. after re-infection	0.537	0.47	1.14	
XXXI	2	4 yrs.	0.475	0.42	1.14	
I	2	3 yrs., 9 mos.	0.432	0.27	1.60	Exposed
XXIV	2	3 yrs., 8 mos.	0.261	0.24	1.10	
XIX	1§	2 yrs., 8 mos.	0.149	0.18	0.82	
XXX	2	2 yrs.	0.235	0.21	1.14	
XI	2	9 mos.	0.805	0.26	3.12	Exposed
XI-a	2	10 wks. after re-infection	0.738	0.19	3.84	
XXV	2	5 mos.	0.581	0.42	1.38	
XXII	2	3 mos.	0.438	0.41	1.08	
XXIX	2	2 mos.	0.895	0.48	1.88	
XXXII	2	1 mo.	2.385	0.44	5.37	
XX	2	Not known‖	0.765	0.29	2.66	Exposed
XVIII	2	Not known¶	0.838	0.51	1.65	

* Four of these sera were obtained through the courtesy of Dr. C. M. Downs, University of Kansas, Department of Bacteriology, Lawrence, Kansas; and three from Dr. Carl Larson, National Institutes of Health, Bethesda, Maryland.

‡ Individuals were considered exposed who were in contact with the organism to the extent that caused infection in non-immune persons.

§ Stored 6 weeks at 0–4°C. before testing; complement not absorbed.

‖ Vaccinated and exposed for 2 years previous to this analysis; postinfection clinical diagnosis.

¶ Vaccinated previously, and exposed for 6 months previous to this analysis; postinfection clinical diagnosis.

The results presented in Table II show that there is measurable, specific, antibody present in the sera after vaccination, but that the amount of antibody is not a function of the amount of vaccine administered. There is also apparent a decrease of antibody when no further injections of antigen are made.

The results presented in Table III show that there is a tendency for more

antibody to be present in the serum of individuals who have recovered from tularemia within the preceding year than in those who had the disease several years previously.

Comparing Tables II and III the following conclusions may be drawn: the serum from a recovered person contains more antibody than does serum from a vaccinated person; the ratio, A/G, is higher in serum from a recovered person; in some instances, the amount of antigen which combines with a unit volume of serum is about the same, regardless of the previous immunizing experience of the person.

A decrease in both the ratio and the amount of antibody following reinfection is exemplified by sera X-a and XI-a. In both there is a significant decrease in A and R when compared to the previous analysis. Using serum XI-a, there was also an increase in the total amount of antigen (from 3.12 mg. N to 3.83 mg. N) which combined with the antibody which the serum contained following reinfection.

DISCUSSION

In the analyses in this study, a low A/G ratio was found in comparison with those observed in comparable studies on other antigen-antibody systems. Kabat (13) has observed low ratios in the sera of persons immunized with blood group A substances, a fact which he attributes to the presence of non-reactive material in the antigen preparation. The preparation used in this study was antigenically homogeneous, as evidenced by its consistent behavior as a single antigen in precipitin tests with animal and human sera. A more precise method of preparation might yield a more active antigen; in fact, several preparations were compared seriologically using goat serum, and it was found that there was a variation in their reactivity. There was also a small amount of insoluble material present after lyophilization, and the reactivity of the solution remained unchanged after removal of this material by centrifugation in a Sorvall high speed centrifuge.

Amounts of precipitable antibody in sera from persons recovered from tularemia varied from 0.149 to 2.385[1] mg. N per 10 ml. serum; in sera from vaccinated persons, from 0.024 to 0.145 mg. N per 10 ml. serum. These values are similar to those found by Heidelberger *et al.* (14–16) in the human response to the pneumococcus, which were 0.025 to 0.245 mg. N per 10 ml. in the case of the previously infected person, and 0.025 to 0.145 mg. N per 10 ml. in the case of the vaccinated person. Kabat has found N values of 0.015 to 0.030 mg. N per 10 ml. serum in persons vaccinated with meningococcal polysaccharide (17); and values from 0.003 to 0.060 mg. N per 10 ml. in persons vaccinated with human blood group substance (13).

A comparison of the amount of antibody and the amount of antigen with

[1] From a patient with acute tularemia, 5 weeks after onset of symptoms.

which it combined in sera of vaccinated as contrasted to recovered persons, all of whom were subsequently exposed to an extent which provoked disease in normal persons, is of interest. Sera of the two vaccinated, exposed individuals (Nos. II and XXIII, Table II) had no more than one-half the quantity of antibody which the four recovered, exposed persons had (Nos. I, XVII, XX, and XXI, Table III); but the A/G ratio in the sera of vaccinated persons was only one-half as large as that from recovered persons, and the antibody in these sera combined with an amount of antigen similar to that combined by sera from recovered persons.

In an analysis made 10 weeks after a clinical diagnosis of reinfection, (serum XI-a, Table III), the amount of antibody had decreased, while the amount of antigen with which it combined had increased. Presumably the individual would have a higher level of immunity because of the stimulus of further antigen.

If the function of circulating antibody in protecting an individual upon exposure to disease is dependent on the ability of the antibody to assist in defending the body against the invasion of antigen, the value of that antibody *in vivo* could reasonably be a function not only of the quantity of antibody, but of its combining capacity as well. This hypothesis is substantiated in the disease tularemia by the work of Foshay *et al.* (7) in which a close correlation was shown between the protective capacity of immune goat and horse sera for white rats, and the antibody as measured by the amount of polysaccharide neutralized.

If this concept of immunity in tularemia is tenable, the efficacy of vaccination against tularemia might better be determined by a method which measured the amount of antigen with which a given amount of antiserum would combine, rather than the amount of antibody alone.

SUMMARY AND CONCLUSIONS

A polysaccharide antigen, prepared from a virulent strain of *Past. tularensis* by phenol extraction, was employed in assaying the antibody in sera from persons recovered from infection with, or vaccinated with *Past. tularensis*, using the serological technique of quantitative precipitation.

Antibody levels in sera of individuals vaccinated with or recovered from infection with *Past. tularensis* were found to resemble those observed in similar studies by other investigators, such as the antibody response to pneumococcal antigen, meningococcal polysaccharide, and blood group substances.

It is suggested that the value of actively or of passively acquired antibody to an individual exposed to tularemia is dependent not on the amount of antibody alone, but also on the amount of antigen with which the antibody is capable of combining.

The author wishes to thank Dr. A. G. Wedum and Dr. A. M. Griffin for their valuable advice during the course of this work; and Agnes C. Baldwin for her able technical assistance.

BIBLIOGRAPHY

1. Foshay, L., Hesselbrock, W. H., Wittenberg, H. J., and Rodenberg, A. H., *Am. J. Pub. Health*, 1942, **32**, 1131.
2. Foshay, L., *Medicine*, 1940, **19**, 1.
3. Downs, C. M., *J. Infect. Dis.*, 1932, **51**, 315.
4. Larson, C. L., *Pub. Health Rep., U. S. P. H. S.*, 1945, **60**, 725.
5. Francis, E., and Felton, L. D., *Pub. Health Rep., U. S. P. H. S.*, 1942, **57**, 44.
6. Larson, C. L., *Pub. Health Rep., U. S. P. H. S.*, 1947, **62**, 1793.
7. Foshay, L., Ruchman, I., and Nicholes, P. S., *J. Clin. Inv.*, 1947, **26**, 756.
8. Palmer, J. W., and Gerlough, T. D., *Science*, 1940, **92**, 155.
9. Nicholes, P. S., unpublished Ph.D. thesis, University of Cincinnati, 1946.
10. Synder, T. L., Penfield, R. A., Engley, F. B., Jr., and Creasy, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 26.
11. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 563.
12. Heidelberger, M., and MacPherson, C. F. W., *Science*, 1943, **97**, 405.
13. Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, 1945, **82**, 207.
14. Heidelberger, M., and Anderson, D. G., *J. Clin. Inv.*, 1944, **23**, 607.
15. Heidelberger, M., MacLeod, C. M., Kaiser, S. J., and Robinson, B., *J. Exp. Med.*, 1946, **83**, 303.
16. Heidelberger, M., MacLeod, C. M., Hodges, R. G., Bernhard, W. G., and Di Lapi, M. M., *J. Exp. Med.*, 1947, **85**, 227.
17. Kabat, E. A., Kaiser, H., and Sikorski, H., *J. Exp. Med.*, 1944, **80**, 299.