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AUTHORITY

smufd, d/a ltr, 14 feb 1972

TRANSLATION NO. 1330

DATE: 31 March 1965

DEPARTMENT OF THE ARMY
Fort Detrick
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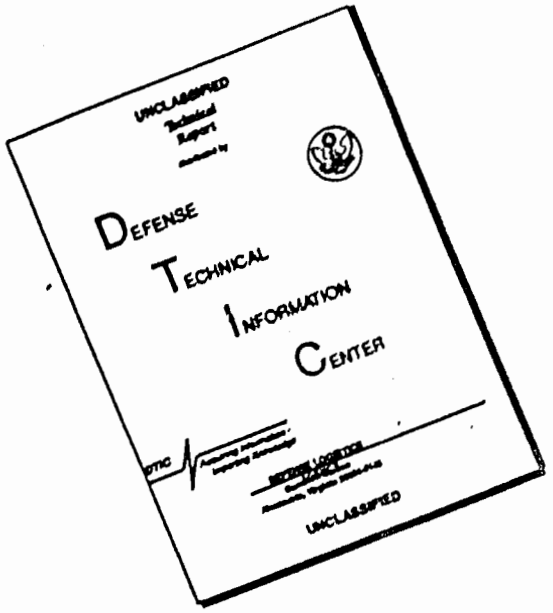

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CONTRIBUTION TO THE SEROLOGY OF TULAREMIA

Zeitschrift fur Bakterologie
(Journal of Bacteriology),
No 181, 1961, pp 80-102

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Hemagglutination, Agglutination, Para-Agglutination and Agglutination Absorption

Contains 4 Illustrations

Received 28 August 1960

Dedicated to Professor Dr. J. Kathe on his 80th Birthday

Tularemia diagnosis today is achieved primarily by means of serological demonstration, since the demonstration of a triggering agent is difficult and many times promises results only if a sensitive test animal is vaccinated with the test material involved. Among these serological methods agglutination has been most widely accepted since antigens are easily produced and relatively easily preserved. The performance capacity of the agglutination reaction has been established by the fundamental research conducted by Francis and Evans so that little extensive research on the same subject has been produced in later years, (8, 10, 11, 21). The agglutinated antibodies are easily demonstrated in human tularemia, displaying great regularity as of the second week of illness, reaching a peak between the third and twelth week. Only in exceptional cases agglutinated antibodies emerge toward the end of the first week of the disease. The percentage of patients with low and also delayed agglutinin formation is equally small. The agglutinating antibodies may persist for many years, and possibly throughout the person's life.

During the agglutination the "zone phenomenon" may be observed at all times, especially in Brucelle serums (4, 17, 22). It should also be recalled that a sharing of receptors occurs between tularemia bacteria and the bacteria of the Brucella group, especially the B. abortus, so that on the one hand specific tularemia serums agglutinate Brucella antigens, while on the other hand tularemia bacteria may be agglutinated also by Brucella serums, however, the reactions in the latter case are substantially weaker than in the former. Serological crossovers with other micro-organisms so

far have never been observed.

Other serological demonstration methods include precipitation (1) on which, however, no extensive research has been carried out, the flocculation test suggested by Hunter (13), and the complement binding reaction (20). More recently, several authors have shown that hemagglutination may become important in the diagnosis of tularemia (2, 5, 7, 26). Wright and Feinberg (26) observed partial hemagglutination titer among convalescents in excess of 1:5,000, and clearly defined prozones up to and including 1:80 were noted in serums with particularly high antibody content. Normal serums, under the same conditions, did not trigger any agglutination of the erythrocytes treated with bacterial polysaccharides. Animal immunology serums reached titer values up to 1:40,000. No correlation could be established in comparing hemagglutination and agglutination titers. Since the titer ratio hemagglutination/agglutination appears to be somewhat higher in convalescents as compared to vaccinated individuals, greater antibody reactivity following natural infection is assumed toward polysaccharides than following artificial immunization. Charkes (5) provided information regarding the reactions during the infection process. According to the author's investigation, which was conducted exclusively with the serums of infected individuals previously safety-vaccinated, the hemagglutinins reached diagnostically useful levels much sooner than the agglutinins. The culminating point of the reaction occurred approximately during the second month of illness and even years afterward hemagglutinin with a titer content higher than agglutinin could be noted in the serum. This article will also deal with conditions prevailing during a natural infection of individuals who had not received preventive vaccination prior to their illness, with emphasis on the comparison between hemagglutination and agglutination, as well as cross reactivity with the B. abortus. We also added a section on agglutination absorption tests in order to complete the picture.

Methods and Materials

Preparation of Antigens and Immune Serums

A slightly virulent strain of bacterium tularense was used in the antigen preparation, designated Strain 501. (This strain was made available to us by Prof. Maassen of the Robert Koch Institute, Berlin.) The cultures were vaccinated continuously for 14 days and hatched for 48 hours at 37°C thermostatically. The nutrition for the strain and the subcultures used was a modification of cystin-Dextrose-Agar, following the Francis procedure (9). Following the hatching, the cultures were rinsed with a physiological NaCl solution containing 0.3% phenol and subsequently passed through a glass filter, (Schott and Gen. G.3). Next, they were centrifuged for 90 minutes at 4,000 rpm and repeatedly washed. The sediment was collected (5 ml sediment to 20 ml physical NaCl solution), shaken for 30 minutes with glass pearls, and then again passed through the glass filter and ultimately, following testing in a gram preparation, bottled. The bacteria suspension was used as an initial suspension for our tests

following a regulation with respect to a barium sulfate density of 96 + 4 at a minimum storage time of 14 days in a refrigerator. Antigen strain suspensions thus prepared could be kept, according to our experience, up to one year in a refrigerator at + 4°C, without decrease in titer during the agglutination reactions.

Brucella antigen was used as the standard test fluid at the Berlin State Health Office.

To calibrate the antigen a tularemia rabbit serum with a 1:1600 titer an agglutinated abortus Bang serum made by the Behring Works Op. 146 with a 1:3,200 titer was used.

In the manufacture of immune serums, we diluted the initial suspension at a 1:10 ratio with a physiol. NaCl solution and heated it for 20 minutes to 60°C. Rabbits, following their serological preliminary blood tests, received increasing dosages (0.25 ml to 2.5 ml) in 3-day intervals through intravenous injection until they reached the proper titer level. About one week after the last injection the animals had their blood removed by means of heart puncture; the blood thus obtained was centrifuged, deactivated at 56°C, mixed with 1:10,000 Cialit and stored at +4°C.

Agglutination Reaction

The deactivated serum to be tested was prepared in geometric dilution series with a physiological NaCl solution containing 0.3% phenol, so that each tube contained 0.5 ml serum dilution. To this we added 0.5 ml of a previously detitered and calibrated antigen suspension and the result was heavily shaken. Following a 2-hour incubation at 37°C, and an 18 hour storage at room temperature, readings were taken, where the positive reactions were classified from + to + + + +. The additional dilution due to the test fluid was not taken into consideration. In order to obtain constant results for each day of the experiment, a positive and a negative serum from the previous day was also investigated each time.

Hemagglutination Reaction

Soluble bacteria extracts were used as antigen, made from B. tularense by means of phenol/water extraction (14, 25). To dilute the serums we used a buffered isotonic NaCl solution, P_H = 7.3, plus 4% added Rinder (Cortex?) albumen Behring. Washed, concentrated blood particles were obtained from human blood, type 0, rH negative, dissolved in a citrate solution. The citrate blood was centrifuged for 10 minutes at 1,500 rpm, the waste was siphoned off, and the blood particles containing the 4-6 fold buffer solution was washed by alternate centrifugation and siphoning action. After three washings, one more centrifuge cycle was completed in order to obtain a maximum concentration of blood particles. After 24-hour storage at + 4°C, the washed, concentrated blood particles were considered useful.

a) Sensitizing of Blood Particles and Production of the Blood Particle Test Fluid

One mg glyco-lipo proteide 501, or lipo-polysaccharide 501 (14, 15) was dissolved in 2.0 ml of buffered isotonic NaCl solution, mixed with 0.1 ml concentrated blood particles and incubated for 4 hours at 37°C, with 30 minute intervals between heavy vibration cycles. Subsequently, the material was centrifuged for 5 to 8 minutes at 1,500 to 2,000 rpm, the waste was siphoned off and the blood particles were washed three times with approximately 6 ml of Buffer solution. After the resuspension of the blood particle sediment in 50 ml Buffer solution, the blood particle test fluid was considered useful (sensitized blood particle suspension). For control, 2.0 ml of buffered isotonic NaCl solution (instead of the antigen solution) was mixed with 0.1 ml concentrated blood particles, exposed to the same conditions, and also rinsed in 50 ml Buffer solution (non-sensitized blood particle suspension).

b) Serum Absorption

Before the hemagglutination test the undiluted serums were deactivated for 30 minutes at 56°C, and whenever necessary diluted with serum albumen solution and subsequently absorbed in 10 Vol % concentrated erythrocyten for 30 minutes at room temperature. For example, to a 5 ml serum diluation 1:5 0.5 ml concentrated erythrocyten are added. After 8 to 10 minutes of centrifugation at 2,000 rpm the same quantity of erythrocyten was added again and mixed with the serum dilution, and without disturbing the sedimented blood particles, if possible. Again, 30 minutes of absorption and centrifugation at 2,000 rpm were completed. The remaining serum was cleaned of a maximum of antibodies and was suited for test use.

c) Hemagglutination Test

Geometric dilution series were initiated with the absorbed test and control serums in such a way that each tube contained 0.5 ml serum dilution. (In all serum dilutions, only the serum albumenum indicated was used as a dilution fluid.) 0.5 ml of the sensitized blood particle fluid was now introduced in each one of these tubes, the tubes were subsequently incubated and readings were taken every two hours at 37°C. Following heavy vibration and an additional 18-hour storage at room temperature, the final reading was taken. In order to eliminate non-specific reactions, the following controls were used: Control A: 0.5 ml of sensitized blood particle suspension + 0.5 ml of Rinder serum albumen solution. Control B: 0.5 ml of non-stabilized blood particle suspension + 0.5 ml of Ringer serum albumen solution. Control C: 0.5 ml of nonsensitized blood particle suspension + 0.5 ml serum dilution 1:5. For Control A one tube per antigen and for Control B one tube per set each is required. Control C was used in each test serum. In addition to the controls described, one each positive and negative serum was titrated in order to obtain constant results. Critique: macroscopic readings, lateral light, agitation of blood particle sediment. The reaction was classified from + to + + + +. The addition of blood particle test fluid was not taken into account and the titer values, as in the Gruber-Widal reaction.

Blocking Test (4)

The agglutination was carried out as described and read off, and subsequently we introduced 0.1 ml of an anti tularemia serum dilution into each tube, calculated such that a + + + + reaction in the antigen controls could be expected. The blocking test was then read and evaluated after two hours at 37°C, and after an additional 18 hours at room temperature, together with the agglutination reaction.

Coombs Test (4)

A second series similar to the agglutination reaction was completed for the indirect Coombs test. On the following day the bacteria were centrifuged and the bacteria sediment was cleansed repeatedly with a physiological NaCl solution by means of repeated centrifugation. Subsequently, we added one droplet each of anti-human-globulin serum from Behring Works, after the sedimented bacteria had been resuspended in one ml of physiological NaCl solution, using any capillary droplet dispenser. If the bacteria cells tied down antibodies, a find flaking could be observed on the ne xt day (after 18 to 24 hours incubation at 37°C) on the bottom of the tubes. This was read off without agitating the tubes and the reactions observed were classified from + to + + + +.

Absorption With Full Bacteria

The antigen suspensions described in the first paragraph were used during the full bacteria absorption (B. tularense, St 501 and standardized Br. abortus test fluid). In addition, we conducted research on bacteria of other origins as well as living bacteria. The results showed agreement and will, therefore, not be discussed. The serums were absorbed as follows: 0.5 ml of undiluted serum was mixed with 4.5 ml antigen suspension ($BaSO_4$, density 96 + 4), abandoned overnight at room temperature, and allowed to settle out by means of 1 to 1-1/2 hours of centrifugation at a minimum of 3,000 rpm. The remainder was then again mixed with the sediment of 5 ml antigen suspension, heavily agitated, and incubated and centrifuged for the second time for 2 hours at 37°C. The serum now had a dilution rate of 1:10 and was then examined by the methods shown above for any remaining agglutinin.

In the Brucelle serum absorption, it was shown frequently that homologous antibodies were still present in some specimens. In these cases, a third absorption with Br. abortus bacteria was made.

Absorption With Sensitized Erythrocyten

0.5 ml of the compressed sensitized erythrocyten was mixed with 4.5 ml of the anti-serums diluted to 1:10 with albumen buffered kitchen salt solution and abandoned overnight at room temperature. We then repeated the absorption at least twice for 2 hours at 37°C, until no further hemagglutinin antibodies could be found.

Results

Investigation of Human Normal Serums

Two hundred negative Wassermann serums of individuals among whom existing or cured tularemia infections could be ruled out with sufficient assurance bordering on probability were used as control test serums. Table 1 shows the distribution of the non-specific reactions and the range from 1:10 to 1:160. No positive reactions were noted in the hemagglutination as well as in the Br. abortus bacteria agglutination starting at 1:160 and up, while this held true for bacteria tularense agglutination already starting at 1:40.

Table 1

Non-specific Reactions of 200 Negative Human Serums

Titer	Bruc. abortus Aggl. No.	Bruc. abortus Aggl. in %	Bruc. tul. Aggl. No.	Bruc. tul. Aggl. in %	Hemagglutination No.	Hemagglutination in %
Under 1:10	105	52.5	186	93.0	141	70.5
1:10	58	29.5	12	6.0	35	17.5
1:20	20	10.0	2	1.0	15	7.5
1:40	15	7.5	0	-	7	3.5
1:80	2	1.0	0	-	2	1.0
1:160 and up	0	-	0	-	0	-

Of the serums 70.5% had a HAR titer below 1:10 while only 52.5% reacted in the Bruc. abortus agglutination test in the same region. The agglutination with Bact. tularense yielded the best result with 93% of all serums below 1:10.

A more detailed investigation was made regarding which titus may be expected upon recovery from tularemia infections and Brucellose illnesses.

Investigation of Tularemia Patient Serums

With the return of tularemia to the Eiderstedt Kreis in the winter of 1957/1958 we had available, in addition to the serological material of the early 1950/1951 cases, the necessary serums to conduct continuous serological investigations during the illnesses. Thus, in the course of one year we examined and evaluated approximately 150 tularemia serums using the indirect hemagglutination method (tul. antigen gly 501 or Po 501) acting as a sensitizing agent (14, 15) and using agglutination reaction (Bruc. abortus and Bact. tularense) from a serological viewpoint. Among these serums, 19 were from patients who had fallen ill during the 1950/1951 epidemic. Individuals who had been tested experimentally with any tularemia skin diagnosis were not considered in the subsequent data. Tables 2 and 3 show the serological results of some tularemia patients in table form. Figures 1a-c show the distribution of hemagglutination and agglutination reactions of all the 48 cases investigated.

Table 2

Serological Investigation of Some Tularaemia Patients; reciprocal hemagglutination (Tul. antigen) and Agglutination (B. tul. and B. abortus)

Patient	Test	(a) Zeit nach Krankheitsbeginn in Tagen bzw. Monaten										
1. W. P.	(b)	2. Tag	6. Tag	11. Tag	–	22. Tag	–	42. Tag	49. Tag	60. Tag	80. Tag	6. Mon.
	B	0	20	40		640		320	320 ±	320 ±	320 ±	160
	T	C	20	80 ±		1280		1280	1280	1280	1280 ±	640
	HAR	20 ±	160	1280 ±		20000		20000 ±	10000	20000 ±	10000	5120
4. E. K.		4. Tag	7. Tag	12. Tag	20. Tag	–	40. Tag	–	–	89. Tag	–	150. Tag
	B	10	10	10 ±	0		10 ±			10 ±		0
	T	0	20	80	160		320			320		80
	HAR	80 ±	640 ±	1280	2560 ±		5120			5120	(c)	5120
9. W. B.			8. Tag	–	24. Tag	–	–	–	–	–	6. Mon.	12. Mon.
	B		320		320						80	40
	T		10		320 ±						160	80
	HAR		20		2560 ±						640 ±	320 ±
10. K. P.			9. Tag	17. Tag	22. Tag	-	34. Tag	49. Tag	-	90. Tag	–	8. Mon.
	B		0	40 ±	40		40	40		40 ±		20
	T		40	2560 ±	2560		5120	2560		2560		1280
	HAR		640	80000 ±	160000 ±		80000	80000		80000		40000
14. J. K.		13. Tag	16. Tag		29. Tag	-	44. Tag		–	114. Tag	8. Mon.	
	B	80 ±	320 ±		640 ±		160			40	20	
	T	80	2560		2560		2560			320	320	
	HAR	10000 ±	80000		80000		40000			10000	10000	
19. M. B.		14. Tag	-	-	–	38. Tag	–	–	90. Tag	–	9. Mon.	
	B	20 ±				0			0		0	
	T	0				0			0		0	
	HAR	64 ±				640			20000		20000	
24. E. S.			–	–	–	30. Tag	56. Tag	68. Tag	100. Tag	–	9. Mon.	
	B					80	160	160	160		80	
	T					1280	1280	1280	1280		640	
	HAR					40000	40000 ±	10000	10000		5120	

Legend: a) Time after beginning of illness in days or months;
b) Second day; c) 6th month

During the first week hemagglutinating and rather considerably weaker agglutinating tularemia antibodies were produced as early as on the 5th day. In three serums an increase from 0 to 1:20 of the agglutination (B. tularense) could be observed during the first week of the illness, as compared with HAR titer values up to 1:1,280 during the period from the 5th to the 7th day. After 7 days, both the hemagglutination reactions and the agglutination reactions increased substantially, generally reaching a maximum value for agglutination (B. tularense) after the 3rd week of the illness and for the hemagglutination reaction after the 4th week of the illness. The average maximum titers were 1:10,000 for the HAR, and 1:640 for the agglutination (B. tularense), while the actual maximum values were 1:160,000 and 1:5,120, respectively. Following the culminating point, both reactions decreased gradually so that after eight years average values of only 1:640 for the HAR and 1:60 for the B. tularense agglutination were obtained. In one case, following the infection, only an increase in hemagglutinating antibodies was noted while the agglutination reaction remained negative after nine months.

Table 3

Serological Investigation of 19 Individuals Who Were Tularemia Patients Eight Years Earlier

Lfd. Nr.	Patient	Test* B	Test* T	Test* HAR
30	H. G.	0	40 ±	640 ±
31	A. J.	10	320 ±	2560 ±
32	F. J.	40	160	1280
33	H. L. P.	20 ±	40	320
34	D. J.	0	80	160
35	C. P.	20 ±	80	1280
36	E. R.	10 ±	40	640
37	J. P.	20 ±	80	2560
38	F. R.	0	40	1280 ±
39	J. D.	0	10	80
40	P. Ch.	0	80	640 ±
41	H. G.	0	40	320
42	F. J.	10 ±	80	640
43	R. D.	20 ±	80	640
44	E. B.	0	40	640
45	A. D.	0	40	320 ±
46	H. D.	0	20	160
47	F. D.	20	160	640
48	J. H.	20	160	640

* Test: B = Bruc.-abortus-Aggl.,
T = Bruc.-tul.-Aggl.,
HAR = Hämaggl. mit sens. Erythrocyten. (a)

Legend: a) HAR - Hemagglutination with sensitized erythrocytens

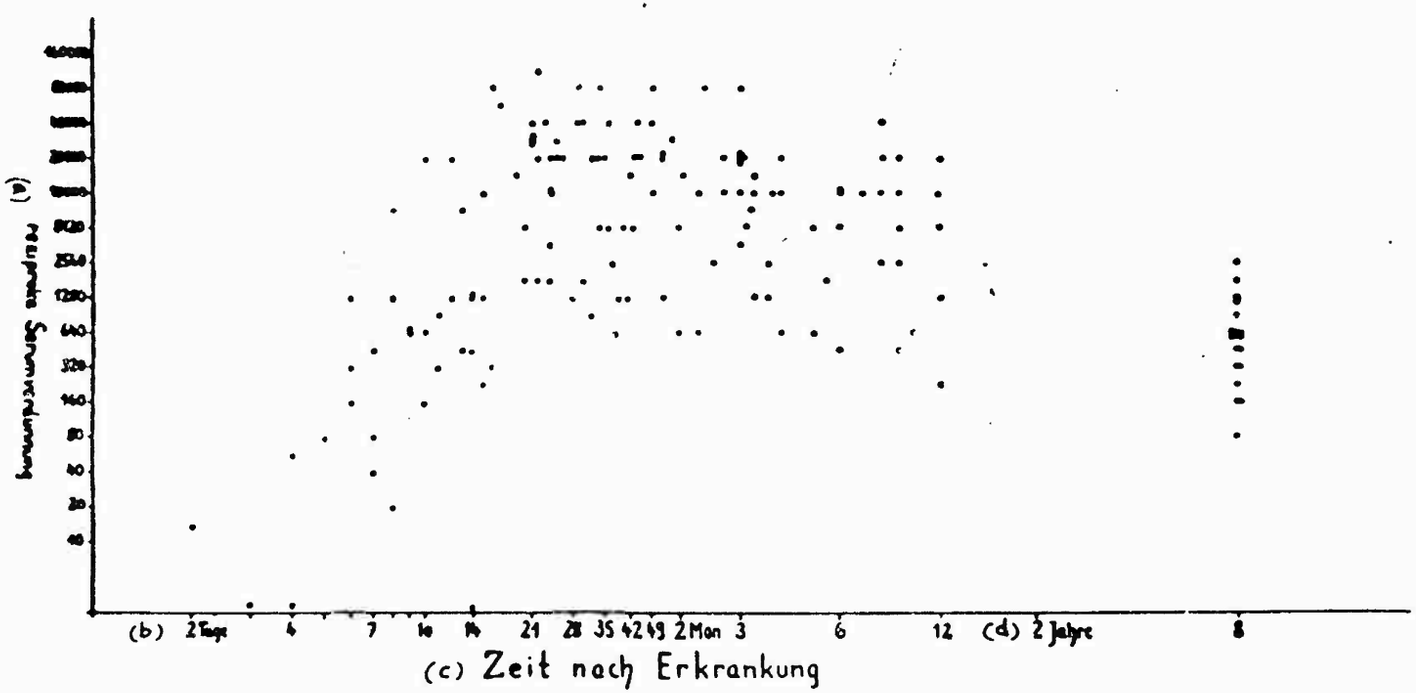


Fig. 1a - Hemagglutination Titer following tularaemia infection (48 cases)

Legend: a) Reciprocal serum dilutions
b) 2 days
c) Time after beginning of illness
d) 2 years

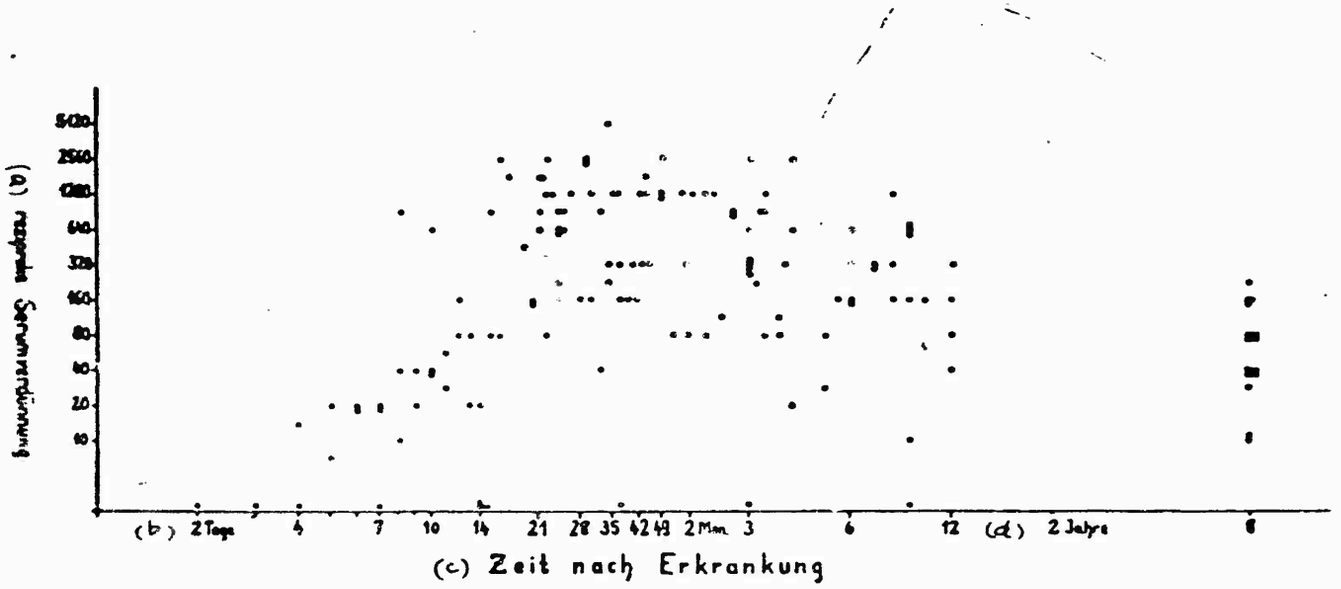


Fig. 1b - B. tularense Agglutination following tularaemia infection

Legend: Same as Fig. 1a

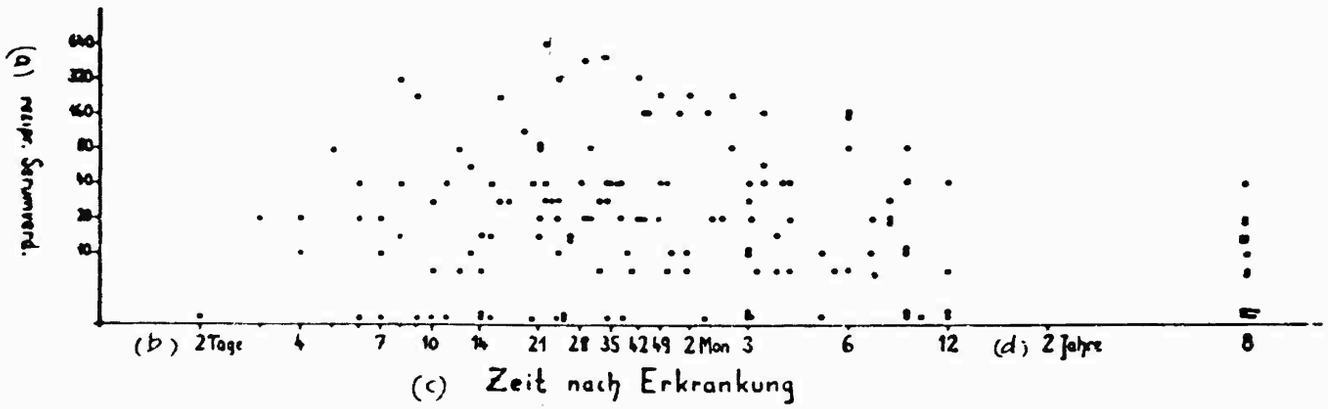


Fig. 1c - Bruc. abortus agglutination titer following tularaemia infection

Legend: Same as Fig. 1a

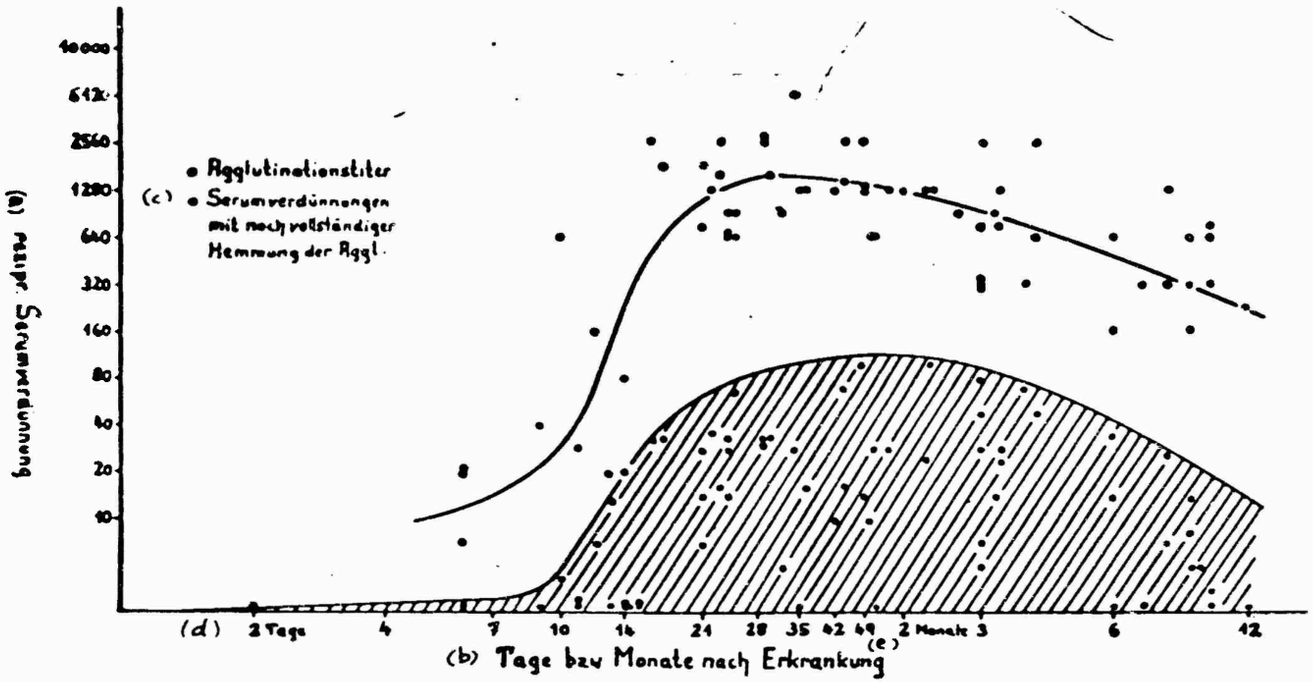


Fig. 2 - Prozones with corresponding agglutination titers (B. tularense) of the Serums from 14 Patients

Legend: a) reciprocal serum dilution;
b) days or months after onset of illness;
c) serum dilutions with complete inhibition of agglutination still in effect;
d) 2 days; e) 2 months.

The absence of agglutination reaction with B. tularense in the small serum dilutions could be observed more frequently. Of the 29 acute cases described the serums of 14 subjects had a clearly pronounced prozone, i.e., at least in the serum dilution of 1:10 the agglutination with Bact. tularense was completely negative. The punctual appearance of prozones with the corresponding agglutination titers of these patients is shown in Fig. 2. Only as the agglutinating tularemia antibodies appeared in increasing numbers (minimum titer for prozone formation: 1:160) an increase in prozones occurred toward the end of the 2nd week of illness, reaching its greatest expansion up to three months following the beginning of the illness -- in some cases up to serum dilutions of 1:80/160 -- and then decreased slowly. During the time interval from 6 to 12 months, negative zones up to serum dilutions of 1:40 could be obtained while the serums of the tularemia patients of the years 1950/1951 contained a zone free agglutination up to the titer indicated. In contrast with Bact. tularense agglutination we observed no prozone formation in the indirect hemagglutination reaction.

Some "prozone serums" as well as a serum sample of patient M.B., who contained no Bact. tularense agglutinin, but positive HAR values, could be tested with the Blocking and Coombs technique (See Table 4).

In all the cases investigated, the prozones were eliminated and thus the presence of incomplete blocking antibodies was demonstrated. The negative agglutination reactions in positive Blocking and Coombs tests in the case of pateitn M.B. point to the fact that incomplete antibodies were exclusively present in the serum. The demonstration method used, however, has only a theoretical value in tularemia diagnosis since indirect hemagglutination methods make available a test which, according to our experience, in all cases leads to zone-free reactions.

In the serums of the 29 acute tularemia cases described, an increased Brucella abortus agglutination titer occurred in 18 cases, however, only in eight cases values exceeding 1:40 were reached and only one serum agglutinated B. abortus bacteria in a 1:640 dilution.

The observation may be of importance that during the early days of the illness an increase in Br. abortus antibody level may occur. Thus, three patients whose serums contained a co-agglutination of Br. abortus, had such a reaction. In the case of most patients, we did not have the samples of the first week of the illness, so that the number may be even greater. Fig. 3 shows the results of the serum tests of patient Th. S. in diagramatic form: the first sample, after nine days, showed a relatively high HAR titer of 1:640 and Br. abortus agglutination titer of 1:320±, while the Gruber-Widal test using B. tularense showed only a low value of 1:20. In the second sample, after 20 days, the B. abortus agglutination reaction had decreased to 1:40 while the B. tularense titer had risen to 1:160. The HAR titer, meanwhile had reached a level of 1:5,120. Accordingly, the curve assumed the same shape as that of other tularemia patients. So-called "paradox" reactions, i.e., a higher agglutination of a heterologous type immediately following the onset of the illness had been known in some infections and under given conditions may be used as an early in-

dicator for preliminary diagnosis, if a Bang infection may be definitely ruled out.

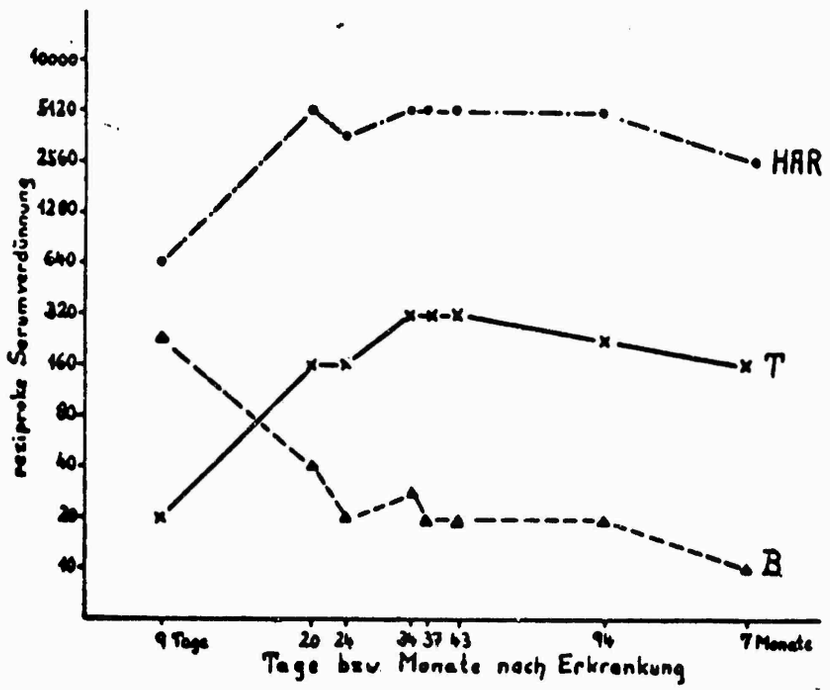


Fig. 3. "Paradox" Reactions in a Tularemia Patient
Legend: Same as in Fig. 2

Examination of Br. Abortus Patient Serum

Since positive hemagglutination reactions were observed in examining animal Bruc. immune serums (using tularemia bacteria extract as antigens) we examined 38 serums of patients with whom the clinical "Brucellose" diagnosis was assumed to be correct. Tularemia infections could be eliminated after additional tests. The serums were tested by means of the indirect hemagglutination method, as in the previous investigations, as well as by means as the B. tularense and B. abortus agglutination method.

Table 5 shows the serological results of a patient whose six blood samples had been received over a two-year period and of whom the time of illness onset had been known. Fig. 4 shows the corresponding curve.

During the first weeks of the illness, in addition to the agglutinating Br. abortus antibody, hemagglutinating and agglutinating B. tularense antibodies were produced. All the maximum values had been reached after four weeks, and the B. abortus titer reached the highest value with 1:10,000. This was followed by the HAR titer with 1:1,280 and B. tularense agglutination titer with 1:160 ±. While the Br. abortus agglutination titer

Table 4

Blocking Test and Indirect Coombs Test of Some Tularaemia Positive Serum.

Patient (a) (Tag nach Erkrank.)	Test	(b) reziproke Serumverdünnungen 5	10	20	40	80	160	320
10. K. P. (17. Tag) (c)	Aggl.	0	0	0	+	+++	++++	++++
	Block.	0	0	0	±	++	+++	++++
	COOMBs	++++	++	++	+	±	0	0
20. I. P. (25. Tag)	Aggl.	0	0	0	0	+	++	++
	Block.	0	0	0	+	++	++	+++
	COOMBs	++++	++++	++++	++++	++++	+++	+++
24. E. S. (30. Tag)	Aggl.	0	0	0	+	+++	++++	++++
	Block.	0	++	+++	++++	++++	++++	++++
	COOMBs	+++	+++	++	++	0	0	0
19. M. B. (90. Tag)	Aggl.	0	0	0	0	0	0	0
	Block.	0	0	0	0	±	++	+++
	COOMBs	+++	+++	+++	++	++	++	+

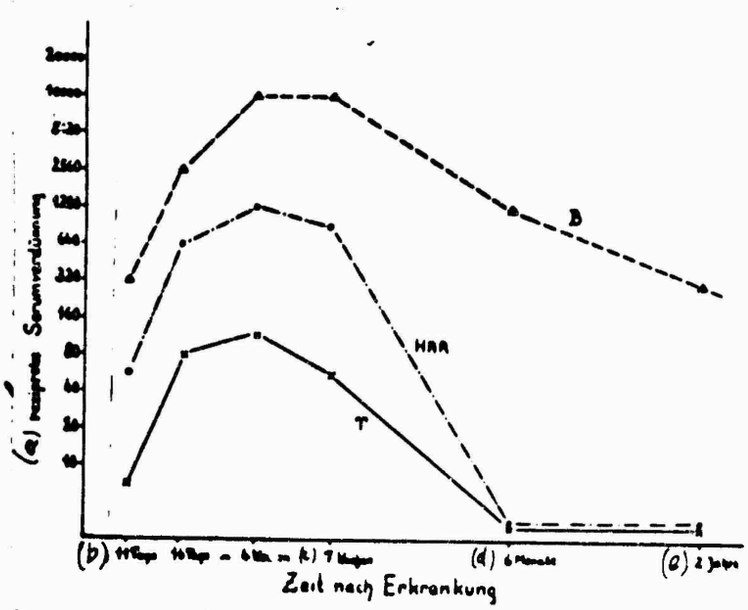
Patient (Tag nach Erkrank.)	Test	reziproke Serumverdünnungen 640	1280	2560	5120	Kontrolle	HAR-Titer
10. K. P. (17. Tag)	Aggl.	++	+	±	0	0	1:80000
	Block.	++++	++++	++++	++++	++++	
	COOMBs	0	0	0	0	0	
20. I. P. (25. Tag)	Aggl.	+	0	0	0	0	
	Block.	++++	++++	++++	++++	++++	1:20000
	COOMBs	+++	++	±	0	0	
24. E. S. (30. Tag)	Aggl.	+++	++	0	0	0	
	Block.	++++	++++	++++	++++	++++	1:40000
	COOMBs	0	0	0	0	0	
19. M. B. (90. Tag)	Aggl.	0	0	0	0	0	
	Block.	+++	++++	++++	++++	++++	1:20000
	COOMBs	±	0	0	0	0	

Legend: a) Day after illness onset;
b) Reciprocal Serum dilution;
c) 17th day

Table 5

Hemagglutination Titer and Agglutination Titer (B. abortus and B. tularense) of Patient L. M. Up To Two Years After Illness Onset

Time after illness onset	Agglutination Br. abortus	B. tularense	Hemagglutination
11th day	1: 320	1: 10 ±	1: 80 ±
16th day	1: 2,560	1: 80	1: 640
4th week	1:10,000	1: 160 ±	1:1,280
7th week	1:10,000	1: 80 ±	1:1,280 ±
6th month	1: 1,280	0	0
2 years	1: 320	0	0



Legend: a) reciprocal serum dilution;
b) 11th day;
c) 7th week;
d) 6 months;
e) 2 years.

Fig. 4. Hemagglutination Reaction (B. tul antigen) and Agglutination Reaction, (B. abortus and B. tularense) of Patient L.M. up to two years after Brucellose illness onset.

still remained at the same level for some time the other reactions decreased. Already, after six months, the values of HAR and B. tularense agglutination had decreased to a titer value below 1:10 while a Br. abortus agglutination titer of 1:1,280 could still be demonstrated! The investigation after two years resulted in a B. abortus agglutination titer of 1:320 for negative HAR and B. tularense agglutination value.

The serological investigations of an additional 32 pateints, of whom only a single serum sample was available, are summarized in Table 6.

Table 6

Hemagglutination Titer and Agglutination Titer (Br. abortus and B. tularense) of an Additional 32 Bang Patients

Serum Nr.	Test* B	T	HAR	Serum Nr.	Test* B	T	HAR
1. P. B.	5120	0	2560 ±	17. L. W.	160	0	20
2. Fr. H.	2560	80	1280	18. A. B.	160	0	20 ±
3. G. F.	2560	10	80	19. H. Bo.	160	0	10 ±
4. K. W.	2560	10	40	20. M. I.	160	0	10 ±
5. H. W.	2560	20	40	21. H. Ho.	160	0	10 ±
6. W. S.	1280 ±	0	10	22. R. H.	160	0	0
7. Fr. B.	1280 ±	10	10	23. M. B.	160	0	0
8. G. B.	1280 ±	10	10 ±	24. E. L.	160	0	0
9. Fr. P.	640	10	20	25. E. E.	80	0	10 ±
10. W. H.	640	0	10 ±	26. U. S.	80	0	10 ±
11. H. R.	640	0	10 ±	27. H. M.	80	0	10 ±
12. H. St.	640	0	0	28. W. N.	80	0	0
13. A. W.	320	0	10	29. L. M.	80	0	0
14. K. Fr.	320	0	10 ±	30. J. Fr.	40	0	20
15. H. Br.	320	0	0	31. R. M.	40	0	0
16. E. P.	320 ±	0	10 ±	32. S. J.	40	0	0

* Agglutination: B = Br.-abortus-Aggl. T = B.-tularense-Aggl.
(a) Hämagglutination: HAR mit Gly 501 bzw. Po 501 als sensibilisierenden Agentien.

Legend: a) Hemagglutination: HAR with Gly 501 and Po 501 as sensitizing agents

In these patients, the beginning of the illness could not be determined accurately, however, it may be assumed that some of them had been infected several years ago, while the patients P.B. (No. 1) and Fr. H. (No. 2) were in acute states of illness.

The titers of the hemagglutination reactions always remained below the values of the Br. abortus agglutination reactions -- the same as among the animal Br. abortus immune serums. If we include in these observations the reactions of patient L.M. described above, a hemagglutination titer of about 1:1,280! could be observed in three cases with a correspondingly high Br. abortus agglutination titer (above 1:2,560) while in one case, no B. tularense agglutination occurred and in the other two values of 1:80 to 1:160 were reached. Three additional serums (No. 3, 4 and 5, Table 6) showed an HAR titer of 1:40 to 1:80 and a B. tularense agglutination titer of 1:10 to 1:20 for a Br. abortus agglutination titer of 1:2,560. All additional values of the HAR and B. tularense agglutination may be con-

Table 7

Agglutinin Absorption Reaction of Tularaemia Anti-serum

Serum	Test Prior to Absorption		Following Absorption with B. tularense	Following Absorption with Br. abortus
1. 501 b immune serum from rabits following injection with heatdeadened Tul. Bact., St. 501	B	1: 320	0	1 0
	T	1: 1,280	0	1: 1,280
	HAR	1:10,000	0	1:10,000
2. 399, immune serum from dog after injection of heat deadened Tul. Bact. St. 501	B	1: 80	0	0
	T	1: 1,280	0	1: 1,280
	HAR	1:40,000	0	1:40,000
3. 235, Case E.S., human serum (taken about 2 months after illness onset)	B	1: 160	0	0
	T	1: 1,280	0	1: 1,280
	HAR	1:10,000	0	1:10,000
4. 208, Case M.B. human serum (taken 1 mo. after illness onset)	B	0	0	0
	T	0	0	0
	HAR	1: 640	0	1: 640
5. 62, Case T. S., human serum (taken 9 days after illness onset)	B	1: 320 ±	0	0
	T	1: 20	0	0
	HAR	1: 640	0	1: 640
6. 337, Case F. S., human serum (taken 10 days after injection of Gly 501)	B	1: 40	0	0
	T	1: 20	0	1: 20
	HAR	1: 2,560	0	1: 2,560
7. 404, Case E. P., human serum (taken 10 days following injection of Po 501)	B	1: 20	1:20	0
	T	0	0	0
	HAR	1: 320	0	1: 320

sidered insignificant.

In three out of 33 cases, therefore, a high, and in another three cases, a low co-agglutination of erythrocytes occurred, which had been sensitized with Bact. tularense extractive materials. According to our tests, therefore, especially in the acute state of Brucellose, we may expect hemagglutinating B. tularense antibodies.

Agglutination Absorption Tests

In seven tularemia and four Br. abortus anti-serums, the B. tularense and the Br. abortus were caused to absorb the agglutinin. The serums were obtained following the tularemia or Brucellose illness onsets or following the unofficial immunization of humans (14) or animals (beef, dog, and rabbit).

Table 7 shows the results of the investigations with various tularemia antiserums. The tularemia bacteria, in all cases, were able to absorb the agglutinin for B. abortus, B. tularense and the hemagglutinizing tularemia antibodies, although no B. tularense agglutinin was present, as in serums 208 (4) and 404 (7). In the latter serum, however, the Br. abortus agglutinin could not be removed by means of the B. tularense. Since the patient (female) had a bisic titer already before the injection of the lipo-polysaccharide 501, the lack of change after the absorption process could be attributed to the fact that no "true" co-agglutination was involved as in the other tularemia antiserums.

Only the Br. abortus agglutinin was removed due to absorption with Br. abortus, while tularemia agglutination and hemagglutination yielded unchanged values after the absorption process.

The results of the absorption reactions of Br. abortus antiserums are shown in Table 8: by means of the Br. abortus bacteria, all agglutinizing and hemagglutinizing antibodies were removed from the serum. Only the agglutinizing and hemagglutinizing tularemia antibodies were removed by means of absorption with B. tularense.

Human and animal serums yielded the same reactions in the previous investigations, therefore, we investigated the absorption capability of sensitized erythrocytes only on rabbit serums. The sensitivation of erythrocytes and their continued treatment occurred in the same manner as that described for hemagglutination. We continued to perform double tests, i.e., sensitized erythrocytes were absorbed with both Gly 501 and Po 501. The results coincided.

Table 9 shows the reactions of a tularemia and Br. abortus antiserum, both before and after the absorption. The erythrocyte sensitized with B. tularense extracts eliminated all agglutinizing and hemagglutinizing antibodies, including the Br. abortus agglutinin in the tularemia antiserums. In contrast, only the agglutinizing and hemagglutinizing tularemia antibodies were removed from the Br. abortus antiserums, while the Br.

Table 8

Agglutinin Absorption Reaction of Br. abortus Antiserums

Serum	Test prior to absorption		Absorbed with B. tularense	Br. abortus
1. B 1, immune serum from beef	B	1: 2,560	1: 2,560	0
	T	1: 80	0	0
	HAR	1: 160	0	0
2. B 4, immune serum from rabbit	B	1: 5,120	1: 5,120	0
	T	1: 20	0	0
	HAR	1: 80	0	0
3. 246, Case L.M. Human serum (4 wks. after infection with Bruc.)	B	1:10,000	1: 10,000	0
	T	1: 160±	0	0
	HAR	1: 1,280	0	0
4. 439, Case P.B. human serum (after infection with Bruc.	B	1: 5,120	1: 5,120	0
	T	0	0	0
	HAR	1: 2,560	0	0

Table 9

Reactions According to Agglutinin Absorption of Two Rabbit Immune Serums With Sensitized Erythrocytes

Serum	Test	Prior to Absorption	Following Absorption
501 b, tularemia immune serum	B	1: 320	0
	T	1: 1,280	0
	HAR	1:10,000	0
B4, Bruc. abortus immune serum	B	1: 512	1:512
	T	1: 20	0
	HAR	1: 160	0

abortus agglutinin remained in the serum.

Therefore, by absorption with homologous antigens all agglutinins (including the heterologous ones) are removed and by absorption with heterologous antigens only the heterologous agglutinins are removed while reactions with tularemia bacteria cells and sensitized erythrocytes qualitatively match the absorption antigens.

Discussion

The absorption of polysaccharide containing bacteria antigens to erythrocytes and the agglutination of erythrocytes sensitized by means of homologous antibodies in recent years has gained considerably in importance (3, 6, 12, 16, 18, 19, 23). Furthermore, erythrocytes, sensitized

by means of polysaccharide extract from tularemia bacteria may be agglutinized by means of homologous antiserum (1, 2, 5, 7, 14, 15, 26).

Comparing the agglutinin and hemagglutin in titers it may be shown that the HAR titers obtained on the average are about three to seven steps higher than the agglutinin titers. The hemagglutinin titers may reach values of 1:10,000 and higher in acute cases, while serums of preventively vaccinated and serums of individuals infected with tularemia bacteria extract displayed lower hemagglutinin titers (5, 14). Although the curves of both reactions are approximately parallel, no relation can be established between the intensity of the reaction in the individual case.

The early occurrence of hemagglutination titer is also of special interest. Thus, in all cases in which the serums were removed during the acute phase of the illness, a serological diagnosis could be made earlier by hemagglutination than by direct agglutination. Thus, it was possible to prove antibody existence already starting on the fourth day of the illness, at a time when agglutination alone would have yielded no definite results. In no case the agglutinin titer was detected before the hemagglutinin titer. Furthermore, it should be noted that the hemagglutinizing antibodies reach a higher value faster than the agglutinins. Of 20 human tularemia infections during which it was possible to remove serums during the first two weeks of the illness, in 18 cases, hemagglutination titers of 1:40 to 1:10,000 could be observed, and only in two cases the reaction continued negative during this period of time. Agglutinizing antibodies, however, in seven cases continued negative, while the remaining 13 tests showed low titer values of 1:20 to 1:80, (only in one case an agglutination titer of 1:1,280 could be detected at the end of the second week of the illness). In contrast, Charkes points out that upon examining 56 patients during the first 14 days of their illness, positive hemagglutination values were noted in only about 40% of them.

Hemagglutination has specific characteristics. Only in Brucella antiserums an increase in tularemia hemagglutination is noted which, however, following the fading away of the acute infection, in most cases decay rapidly while the Brucella agglutination titer decreases only slowly. In other cases, the Brucella antiserums always had a higher Brucella agglutination titer for the same hemagglutination and bacterium tularense agglutination titer. Serums from patients who had other bacterial infections showed no hemagglutination.

If any doubts exist in the evaluation of the findings, the agglutinin absorption tests have proved very useful. With regard to the absorption of bacterial agglutinins, our results agree with those obtained by Francis and Evans. However, in addition, we were able to show that the hemagglutinizing antibodies act in the absorption tests like agglutinizing antibodies.

While prozones were observed in tularemia infections during the writing of this report, no case has become known in which the agglutination reactions were completely absent. Nevertheless, in one case we were able to make a diagnosis with only indirect hemagglutination methods.

Coomb's test and blocking tests showed the existence of univalent antibodies. The prozone formation of the material investigated may be considered extraordinarily high. Almost 90% of all the cases investigated were characterized by the presence of prozones in the lower serum dilutions, while the titers of the serums were 1:160 or higher. Blocking of the agglutination reaction could be verified up to a serum dilution of 1:160 for sufficiently high agglutination titer. Since evidently prozones could only be observed in higher titers, it becomes understandable that in addition to the acute illness hardly any blocking of the agglutination could be noted. In contrast with Wright and Feinberg no zone formation could be observed during the hemagglutination reaction.

Summary

1. The examination of about 150 serum specimens from different stages of this disease proved that hemagglutinating antibodies may be demonstrated from the 5th day on. In tularemia infections the rise of hemaglutinating antibodies nearly parallels that of the antibodies agglutinating B. tularense. The former usually show titers three to seven dilution steps higher than the latter, but this relation is not a general one.

2. The hemagglutination reactions never showed a prozone which was found in 50% of the bacterial agglutinations in one of the cases tested.

3. During the first days of illness in three out of 29 cases heterologous bacteria, e.g. Br. abortus, have been agglutinated to higher titers than B. tularense. The hemagglutinating titers always surpassed those of the bacterial agglutinins whether B. tularense or heterologous bacteria were used as antigens. Thus, an equivocal serological diagnosis is always possible. This applies also to later stages of the illness. In approximately 50% of the cases Brucella abortus was also agglutinated by the sera.

4. Cross reactions with Br. abortus immune sera occur, but do not cause diagnostic difficulties. In cases of Br. abortus infections, especially during the acute phase, hemagglutinating tularemia antibodies may develop. The hemagglutinating anti-tularemia antibodies never surpassed the titers of Br. abortus agglutinatins.

5. In the absorption test specific hemagglutinins behave like bacterial agglutinins.

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