PASSIVE PROTECTION EXPERIMENTS WITH BRUCELLA ANTISERA

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(With 1 Figure in the Text)

Passive protection experiments with brucella antisera are relatively few in comparison with the numerous studies on active anti-brucella immunity.

Holth (1911) was the first to show that horse and rabbit antisera protected mice against lethal challenge with living broth cultures. His results were confirmed by Priestley & McEwen (1938), who also demonstrated that antisera prepared with living or dead organisms were equally effective. Olitzki & Oren (1950) used the mucin technique in passive protection experiments with sera obtained from vaccinated and diseased cattle. Live & Giuliani (1953) showed that sera of cattle vaccinated with ether-killed *Brucella abortus* in adjuvants afforded better protection to mice than the sera of animals immunized with living *Br. abortus* strain 19.

The present work was prompted primarily by the need for evaluating immunologically the effect of living streptomycin-dependent *Br. abortus* vaccines in men (Olitzki & Sulitzeanu, 1953). Although a rise of agglutinin titre was observed following the inoculations, the presence of agglutinins could not be considered as proof of increased resistance to infection (Elberg & Silverman, 1950). Recourse was therefore made to the measurement of the protective power of the sera of vaccinated people.

Previous studies on the protective effect of sera against brucella have been based on the percentage of animals surviving the lethal challenge doses, with or without mucin. Such severe tests have lacked sensitivity and required comparatively large numbers of animals to obtain statistically significant differences. In the work described below the spleen counts technique has been used in an attempt to increase the sensitivity of the test. This technique has been used in anti-brucella immunity measurements by De Ropp (1945), Olitzki & Szenberg (1953) and Olitzki & Sulitzeanu (1954). It has been based upon a comparison of bacterial counts in spleens of immune and normal animals.

MATERIALS AND METHODS

Animals. White mice of both sexes, weighing 20–25 g. (8 weeks of age), were used.

Cultural media. The bacteria used for challenge and stock cultures were grown on Trypticase Soy agar (Baltimore Biological Laboratory, Baltimore, Md.) to

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which 3 % glycerol and 1 ml./l. of a 0·01 % thiamin solution were added. This medium will be designated as 100 % BA (100 % brucella agar).

The spleen suspensions were cultured on agar plates, prepared by mixing 10 ml. 100 % BA with 90 ml. nutrient agar, the latter containing glycerol and thiamin in the stated proportions. This medium, designated as 10 % BA, was used in order to save the more expensive Trypticase Soy Agar. Comparative counts on these media showed that the 10 % BA was at least as good as the 100 % BA and sometimes even better. 100 and 10 % brucella broth (BB) were prepared in a similar manner from Trypticase Soy Broth.

Bacterial strains. Br. abortus 19 (A 19) was the immunizing strain, and *Br. abortus* 2308 (A 2308), obtained from the National Institute of Health, Bethesda, Md., was the challenge strain. Stock cultures were stored in the ice box and transferred at 6-month intervals.

The cultures were periodically tested for smoothness by the crystal violet technique of White & Wilson (1951). For this test 2–1 agar was used (Elberg, Herzberg, Schneider, Silverman & Meyer, 1951). Difficulties were encountered in the preparation of this medium. The use of a technical batch of glycerol made the smooth colonies appear deep blue violet, instead of the usual light blue-green colour of the colonies of the same inoculum grown on agar prepared with chemically pure glycerol.

In the course of routine transfers A 2308 lost its virulence, but this was restored after two passages in mice. To avoid repetition of similar accidents the brucellae were cultured on slants prepared in large test tubes (120 × 30 mm.) and the tubes, stoppered with rubber corks, were stored in the refrigerator (4° C.). In this way a large stock of a strain with proved virulence was available, from which transfers could be made when required.

Agglutination technique. Dilutions of sera were prepared in buffered saline (pH 7·2) in a volume of 0·5 ml., and 0·5 ml. antigen was added. The tests were incubated at 37° C. in a water-bath for 24 hr. 50 % end-points were determined according to the method recommended by the Joint FAO/WHO Expert Panel on Brucellosis (1951).

The antigen was prepared according to the technique of Carrère & Quatrefages (1950), and standardized against a standard brucella serum kindly supplied by Dr A. W. Stableforth, Veterinary Laboratory, New Haw, Weybridge, Surrey, England.

Preparation of suspensions for inoculation. A 48 hr. culture of the challenge strain was washed off with 10 % BB and the suspension adjusted to 50 % light transmission in a Coleman Jr. spectrophotometer, at 6500 A wavelength, against a blank of the same broth in which the bacteria were suspended. This suspension contained about 2×10^9 viable organisms of A 2308. This number was not constant, however, and variations were sometimes encountered. Suspensions of A 19, of the same optical density, contained 3×10^9/ml. and were more constant in number.

The required dilutions were prepared from the above suspensions. Inocula were administered intraperitoneally, in a volume of 0·1 ml. All inoculations were checked by plate counts.

The challenge dose was $2–6 \times 10^4$ bacteria. This concentration was critical and it had to be carefully controlled. With higher doses, the spleen counts of the immune animals were too high. With lower doses, the spleen counts of the controls were too low. In both cases the Protection Index (see below) was adversely affected.

Technique of the protection test. Both fresh and stored sera were used. To avoid errors due to the presence of varying quantities of complement, all sera were first inactivated for 30 min. at 56° C. The protection measured was thus entirely due to specific protecting antibodies. 0·1 ml. of serum, diluted in buffered saline as desired, was inoculated intramuscularly into the left thigh of five mice. A control group of five untreated mice was included with each experiment. During the early stages of this work a second control group was inoculated with normal serum, but this practice was discontinued after it was ascertained that normal sera did not give any protection above a certain dilution.

Eighteen hours after serum inoculation the animals were challenged.

Table 1. *Relation between logarithms of arithmetic means and logarithmic means*

	Spleen counts			
Days after inoculation	Arithmetic means (1)	Log of arithmetic means (2)	Logarithmic means (3)	Difference (2 – 3)
1	$6·8 \times 10^4$	4·8325	4·6475	0·1850
7	$5·3 \times 10^5$	5·7243	5·4254	0·2989
12	$1·9 \times 10^6$	6·2900	5·8664	0·4236
27	$1·1 \times 10^6$	6·0294	5·7829	0·2465
Mean		5·7190	5·4305	0·2880

Mean difference (2 – 3) = 0·288 or approx. 5 % of the arithmetic mean.

*Technique of spleen counts.** Animals were killed with ether. The spleens of each experimental group (normally five mice) were removed aseptically and thoroughly ground together with glass sand in porcelain mortars. On occasion counts of single spleens were performed. To the pool of five spleens 50 ml. 10 % BB were added (10 ml. per spleen). 0·2 ml. of this suspension, or of further tenfold dilutions were spread on 10 % BA plates with Drigalski rods. At least three plates were used per group. The plates were incubated for 4–6 days and the colonies counted. No difficulties were encountered in distinguishing between the glistening brucella colonies and occasional contaminants.

Statistical analysis. In calculating standard deviations (S.D.) it was considered more appropriate to use logarithmic values of spleen counts instead of the actual numbers, in order to bring these counts to a more nearly normal distribution.

S.D.'s were accordingly calculated from the logarithms of spleen counts (logarithmic S.D.'s). When comparing spleen counts, however, only logarithms of arithmetic means were available since the spleens from five mice had been pooled.

* With the technique outlined above, one colony on the plate represented fifty organisms in the original spleen ($1 \times 1/5 \times 1/10$, where 1/5 stands for the 0·2 ml. cultured and 1/10 the quantity of broth added per spleen). Since three plates were normally used per group, the original suspension had to contain at least seventeen organisms per spleen in order that a colony might appear.

It was therefore necessary to estimate the means of the logarithms of spleen counts from the logarithms of the arithmetic means. This was done as shown in Table 1, where it can be seen that the former are smaller than the latter, on the average, by about 5 %. This difference was considered small enough to be neglected, and therefore S.D.'s obtained from logarithmic values were used for the purpose of comparing logarithms of arithmetic means.

Protection Index. The logarithm of the ratio

$$\frac{A}{B} = \frac{\text{spleen count of control group}}{\text{spleen count of immune group}}$$

was taken as the measure of the degree of protection (Protection Index—P.I.).*

EXPERIMENTAL RESULTS

The preliminary experiments were performed with rabbit sera.

(1) *Protective effect of normal rabbit sera.* Several tests were performed to determine the protective index of normal rabbit sera. The results are summarized in Table 2 and show that, except for a slight protective effect of undiluted serum, normal sera were inactive against brucella infection.

Table 2. *Protective effect of normal rabbit sera*

Serum no.	Time of autopsy (days)	Challenge dose	Serum* dilutions	Spleen counts 'Protected'	Spleen counts Controls	P.I.
1	2	$5{\cdot}5 \times 10^4$	1/1	$3{\cdot}2 \times 10^4$	$2{\cdot}1 \times 10^5$	0·82
			1/5	$9{\cdot}5 \times 10^4$		0·34
			1/10	$6{\cdot}8 \times 10^4$		0·49
2	7	$2{\cdot}4 \times 10^4$	1/2	$3{\cdot}5 \times 10^4$	$4{\cdot}3 \times 10^4$	0·08
			1/5	$6{\cdot}5 \times 10^4$		0
			1/10	8×10^4		0
3	7	6×10^4	1/10	$3{\cdot}4 \times 10^5$	$6{\cdot}4 \times 10^5$	0·27
4	7	$2{\cdot}5 \times 10^4$	1/10	$1{\cdot}2 \times 10^5$	$3{\cdot}7 \times 10^5$	0·49

* Volume inoculated: 0·1 ml.

(2) *Course of brucella infection in passively protected mice.* In order to determine the optimum time for autopsy, i.e. the time after challenge at which a maximum protective index could be obtained, the course of infection in passively protected mice was followed for a period of 3 weeks. The serum used for immunization was taken from a rabbit immunized with living A 19 and had an agglutinating titre of 1 : 3200. It can be seen from Table 3 that the multiplication of brucellae in the protected animals was checked during the first week after inoculation of the immune serum. After this period there was a sharp rise in the number of organisms in the spleen, approaching the counts in the control spleens by the third week after challenge. The highest protective index was obtained during the first week following inoculation.

(3) *Variability of spleen counts in passively immunized mice.* It was necessary to determine the variability of the spleen counts in order to assess the significance of

* When $B > A$ the P.I. takes a negative value. Also when $B = 0$ its log is infinity. To obviate these difficulties the smallest P.I. and the smallest $\log B$ were taken to be zero. The latter restriction is practically equivalent to defining $\text{P.I.} = \log (A+1) - \log (B+1)$.

differences between spleen counts. Standard deviations were therefore calculated from spleen counts of individual mice. A knowledge of the variability of counts was also required for a final decision on the optimum date of autopsy. Obviously, the ideal time of autopsy should combine a high protective index with low variability.

Table 3. *Course of infection in passively protected mice, challenged with* 6×10^4 A 2308 (*log mean spleen counts in groups of five mice*)

Days after challenge ...	1	4	7	10	20
Immune serum 1/10	2·4472	3·3802	3·0792	4·5051	5·9031
Normal serum 1/10	4·6021	5·4771	5·5315	6·1139	6·6532
Controls	4·4771	5·7782	5·8062	5·9031	6·5441
P.I.	2·03	2·4	2·73	1·4	0·64

Table 4. *Spleen counts of individual mice passively protected with* 0·1 *ml. of immune rabbit serum against brucella and challenged with* 6×10^4 A 2308

	Section after 2 days		Section after 7 days	
Mouse no.	Immune	Controls	Immune	Controls
1	75	4×10^3	350	10^4
2	75	2×10^4	500	$1{\cdot}6 \times 10^5$
3	200	3×10^4	700	2×10^5
4	300	4×10^4	800	$2{\cdot}1 \times 10^5$
5	300	5×10^4	1700	3×10^5
6	400	7×10^4	2100	$6{\cdot}2 \times 10^5$
7	550	1×10^5	2700	1×10^6
8	900	1×10^5	3500	$1{\cdot}7 \times 10^6$
9	1200	2×10^5	5000	—*
10	2700	—*	6000	—*
Mean	670	$6{\cdot}8 \times 10^4$	2300	$5{\cdot}3 \times 10^5$
Log mean	2·5813	4·6475	3·2000	5·4254
Log S.D.	0·5	0·5	0·41	0·695
P.I.	2·07		2·37	
S.E. of P.I.	0·32		0·36	

* Not done.

A group of twenty mice was inoculated with 0·1 ml. of the immune rabbit serum used in section 2, diluted 1/10. They and their controls were then challenged with 6×10^4 brucellae. Ten mice were autopsied at 2 days and ten at 7 days after challenge and the spleens counted individually.

Table 4 reveals that at 7 days the P.I. was higher and the S.E. approximately the same as at 2 days. The data in Table 3 had already shown that the P.I. was highest at 7 days. It was concluded that the optimum time for autopsy was 7 days after challenge.

The standard error (S.E.) of the P.I. was calculated as follows:

$$\text{P.I.} = \log A/B = \log A - \log B,$$

$$\text{S.E.}_{\text{P.I.}} = \text{S.E.}\ (\log A - \log B) = \sqrt{\left(\frac{a^2}{n} + \frac{b^2}{n}\right)},$$

where a = logarithmic S.D. of A, b = logarithmic S.D. of B, n = number of animals per experimental group.

A difference between two P.I.'s larger than 0·72, i.e. twice the S.E., was considered significant. Since the highest obtainable P.I. was of the order of 2·5–3, four levels of protection could, under optimal conditions, be differentiated with this technique, corresponding to approximate observed P.I. values: 0·72, 1·44, 2·16, 2·88.

(4) *Variation of the protective index with serum dilutions.* Fig. 1 shows the results of a typical experiment on the effect of serum dilutions upon the protective index. A P.I. of the same order of magnitude was obtained throughout the dilution range 1/2–1/50. The P.I. fell off with higher dilutions.*

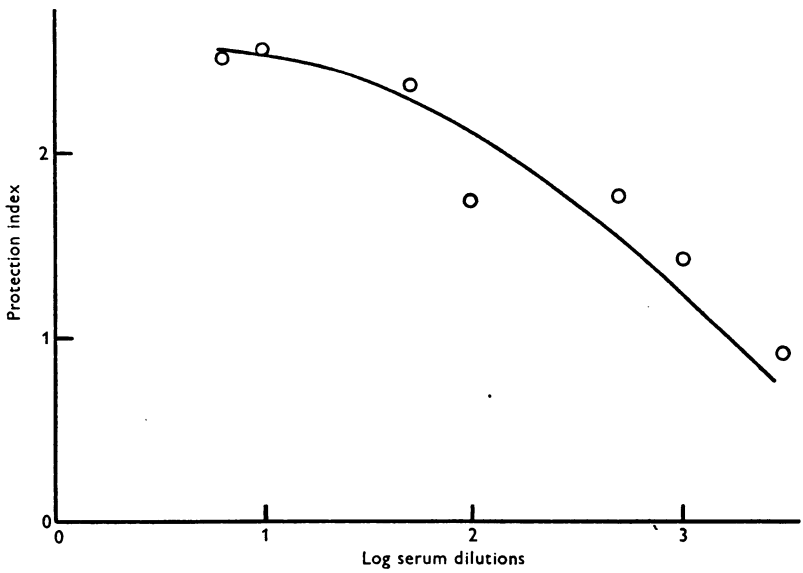

Fig. 1.

(5) *Protection tests with human sera.* The protective effect of normal human sera was first determined. The results, summarized in Table 5, showed that some normal human sera had considerable protective power. Some were active to a certain degree even at the 1/10 dilution.

All sera tested were negative in the agglutination test with brucella antigen, starting with a dilution of 1/10.

Four volunteers were vaccinated with living streptomycin-dependent *Br. abortus* 19. Details on the vaccination schedule have been published elsewhere (Olitzki & Sulitzeanu, 1953).

The sera of these volunteers were tested for protective activity (Table 6). The serum of volunteer no. 1 was tested only at the 1/2 dilution, since his normal serum did not show any protection at this dilution. The others were tested at the higher dilutions, after it became evident that normal sera might be active below the 1/10 dilution. Two samples were taken from volunteer no. 2: the first, on com-

* The experiment reported in this section has been performed at the Veterinary Laboratory, New Haw, Weybridge, in collaboration with Dr L. Jones. The author is grateful to the Director, Dr A. W. Stableforth, and to Dr L. Jones, for permission to publish these results.

pletion of the vaccination schedule (titre 1/1000), and the second 3 months later (titre 1/80). It is seen that the protecting power did not decrease to an appreciable extent during this time.

Table 5. *Protection tests with normal human sera*

Serum no.	Challenge dose	Time of autopsy (days)	Serum dilution and quantity inoculated	Spleen counts		P.I.
				Protected	Controls	
1	5×10^4	10	Undil. 0·1 ml.	$4{\cdot}2 \times 10^4$	$4{\cdot}6 \times 10^6$	2·04
	$2{\cdot}7 \times 10^4$	7	1/2 0·1 ml.	$1{\cdot}3 \times 10^5$	$1{\cdot}4 \times 10^5$	0·03
2	5×10^4	10	Undil. 0·1 ml.	$3{\cdot}2 \times 10^5$	$4{\cdot}6 \times 10^6$	1·16
	5×10^4	10	Undil. 0·2 ml.	$1{\cdot}3 \times 10^5$	$4{\cdot}6 \times 10^6$	1·55
3	5×10^4	10	Undil. 0·1 ml.	3×10^3	$4{\cdot}6 \times 10^6$	3·19
	5×10^4	10	Undil. 0·3 ml.	1×10^4	$4{\cdot}6 \times 10^6$	2·66
4	5×10^4	10	Undil. 0·1 ml.	1×10^4	$4{\cdot}6 \times 10^6$	2·66
5	6×10^4	7	1/2 0·1 ml.	$1{\cdot}1 \times 10^5$	$3{\cdot}5 \times 10^5$	0·50
	6×10^4	7	1/5 0·1 ml.	5×10^4	$3{\cdot}5 \times 10^5$	0·85
6	$1{\cdot}4 \times 10^4$	7	1/5 0·1 ml.	3×10^4	3×10^5	1·00
	$1{\cdot}4 \times 10^4$	7	1/10 0·1 ml.	$1{\cdot}1 \times 10^5$	3×10^5	0·44
7	$1{\cdot}4 \times 10^4$	7	1/5 0·1 ml.	1×10^4	3×10^5	1·48
	$1{\cdot}4 \times 10^4$	7	1/10 0·1 ml.	5×10^4	3×10^5	0·78
8	$1{\cdot}4 \times 10^4$	7	1/5 0·1 ml.	$7{\cdot}5 \times 10^4$	3×10^5	0·60
	$1{\cdot}4 \times 10^4$	7	1/10 0·1 ml.	$1{\cdot}4 \times 10^5$	3×10^5	0·33
9	$1{\cdot}4 \times 10^4$	7	1/5 0·1 ml.	3×10^4	3×10^5	1·00
	$1{\cdot}4 \times 10^4$	7	1/10 0·1 ml.	5×10^4	3×10^5	0·78
10	$1{\cdot}4 \times 10^4$	7	1/5 0·1 ml.	6×10^4	3×10^5	0·70
	$1{\cdot}4 \times 10^4$	7	1/10 0·1 ml.	1×10^5	3×10^5	0·48

Table 6. *Protection by sera of human volunteers vaccinated with living streptomycin-dependent* Br. abortus 19

Serum no.	Aggl. titre	Challenge dose	Date of autopsy (days)	Type of serum	Spleen counts at dilutions stated*			Controls	P.I.
					1/2	1/5	1/10		
1*a*	0	$2{\cdot}7 \times 10^4$	7	Prevac.	$1{\cdot}3 \times 10^5$	—	—	$1{\cdot}4 \times 10^5$	0·03
1*b*	320	$2{\cdot}7 \times 10^4$	7	Postvac.	300	—	—	$1{\cdot}4 \times 10^5$	2·67
2*a*	1000	2×10^4	7	Postvac.	—	—	1800	$2{\cdot}3 \times 10^5$	2·11
2*b*	80	$2{\cdot}7 \times 10^4$	7	Postvac.	1800	—	—	$1{\cdot}4 \times 10^5$	1·90
	80	2×10^4	7	Postvac.	—	—	3400	$2{\cdot}3 \times 10^5$	1·83
3	320	$2{\cdot}7 \times 10^4$	7	Postvac.	2300	—	—	$1{\cdot}4 \times 10^5$	1·78
	320	2×10^4	7	Postvac.	—	—	1500	$2{\cdot}3 \times 10^5$	2·19
4*a*	20	$1{\cdot}3 \times 10^4$	7	Prevac.	1100	—	—	$3{\cdot}6 \times 10^5$	2·52
		$1{\cdot}3 \times 10^4$	7	Prevac	—	700	—	$3{\cdot}6 \times 10^5$	2·71
		$1{\cdot}3 \times 10^4$	7	Prevac.	—	—	$1{\cdot}2 \times 10^4$	$3{\cdot}6 \times 10^5$	1·48
4*b*	40	$1{\cdot}3 \times 10^4$	7	Postvac.	700	—	—	$3{\cdot}6 \times 10^5$	2·71
		$1{\cdot}3 \times 10^4$	7	Postvac.	—	700	—	$3{\cdot}6 \times 10^5$	2·71
		$1{\cdot}3 \times 10^4$	7	Postvac.	—	—	1200	$3{\cdot}6 \times 10^5$	2·48

* Volume inoculated: 0·1 ml.

Serum no. 4 belonged to a laboratory worker who had been working with brucella for some time. This is why the pre-vaccination serum reacted with brucella antigen. The effect of vaccination can be observed only with the 1/10 dilution.

Immune sera nos. 3 and 4*b* were also tested at higher dilutions (Table 7). It is seen that they were active at least up to 1/250 dilution. To compare quantitatively the protective action of two sera, it was therefore necessary to employ several dilutions. Thus, in the given example, the P.I.'s of the two sera differed significantly at the 1/250 dilution only.

Sera of three patients with a certain diagnosis of brucellosis (two of them with positive blood cultures) were available and tested for protective activity. All three gave a high P.I. (Table 8).

Table 7. *Protection by sera of volunteers nos.* 3 *and* 4, *tested at higher dilutions*

Serum no.	Serum dilutions 1/50	P.I.	1/250	P.I.	Controls
3	10^3	2·54	2×10^3	2·24	$3{\cdot}5 \times 10^5$
4*b*	$1{\cdot}6 \times 10^3$	2·34	10^4	1·54	—

Table 8. *Protection by sera of adult patients with brucellosis*

Serum no.	Blood culture	Challenge dose	Agglutination titre	Date of autopsy (days)	Dilution	Spleen counts Patients' sera	Controls	P.I.
1	Pos.	6×10^4	800	7	1/5	$2{\cdot}1 \times 10^3$	$3{\cdot}5 \times 10^5$	2·22
2	Neg.	6×10^4	320	7	1/5	$1{\cdot}4 \times 10^3$	—	2·40
3	Pos.	$1{\cdot}7 \times 10^4$	—	7	1/10	$1{\cdot}4 \times 10^3$	—	2·40
					1/50	$1{\cdot}9 \times 10^3$	—	2·27

DISCUSSION

The present investigation demonstrates the sensitivity and usefulness of the spleen counts technique for the measurement of the protective effect of anti-brucella sera. Maximum protection (highest P.I.) occurred in passively protected mice 7 days after a challenge inoculation. Normal human sera were found to have significant protective power up to 1/10 dilution. Vaccination of human beings with living streptomycin-dependent *Br. abortus* 19 increased the protective power of their sera.

The usefulness of vaccines in the therapy of brucellosis is still a debated question, mainly because their effects can only be judged on the basis of subjective clinical impression. The protection test provides a means whereby it is possible to demonstrate objectively at least one favourable effect, namely an increase of the protecting power of the serum. It would be of interest to test in this way the effectiveness of various vaccines employed in human vaccine therapy. It would seem *a priori* that the living streptomycin-dependent bacteria should be best and they deserve a clinical trial on a larger scale. It might even be found practicable to inoculate people exposed to heavy infection risks, such as veterinarians, laboratory workers, meat plant workers, etc.

The protection obtained with sera of brucellosis patients suggests the possibility of using this test for diagnostic purposes but much additional work is needed to ascertain its diagnostic value.

An unexplained phenomenon is the relative constancy of the P.I. in varying dilutions of human and rabbit sera. Although the highest attainable P.I. is of the order of 5 (10^5 in controls against 0 in immune), no such degree of protection is found. Most P.I.'s have values between 2 and 3 at 1/100 dilution and values remain at this level despite lower dilutions. It might be expected that P.I. would increase with decreasing serum dilutions. However, an increase in serum concentration of 100-fold (from 1/250 to 1/2) is not accompanied by an increase in P.I. It is difficult to account for this phenomenon unless it is assumed that a certain percentage of brucellae are protected from the lethal effects of the immune serum.

No significant correlation is noted between P.I. and agglutinin titres, an observation previously reported by Olitzki & Oren (1950) and Live & Giuliani (1953). This is clearly demonstrated by a comparison of sera nos. 2 and 4 (Table 6). Serum no. 2 has a P.I. of 2·11 and an agglutinin titre of 1/1000; serum no. 4 a P.I. of 2·48 and an agglutinin titre of 1/40.

The antigen involved in protection is most likely a surface antigen, as the protective activity can be removed by absorption with whole bacteria.

The present test is more laborious than that based on lethal challenge doses. On the other hand, it is more sensitive, since four observed levels of protection could, under optimum conditions, be differentiated with only five animals per group. Statistical considerations show that about twenty animals per group must be employed with the death and survival method to reach a comparable degree of sensitivity.

Further work is required, as pointed out by Live & Giuliani (1953), to determine the degree of correlation of the serum protective power with clinical resistance to infection. Should such correlation be found, a sensitive protection test would prove most useful for providing a relatively quick answer to many vaccination problems, which can at present only be solved by tedious and time-consuming work.

SUMMARY

A passive protection test with brucella antibodies, based on the spleen counts technique, has been described. This test has been found more sensitive than that based on lethal challenge.

Normal human sera have been shown to possess marked protecting power.

Protective activity of human sera was considerably increased following vaccination with living streptomycin-dependent brucellae, or after natural disease.

The bearing of these findings on several brucellosis problems has been briefly discussed.

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