Antibodies in Human Brucellosis

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There have been a number of studies of the serological and immunological properties of brucella antibodies in man and animals. Vaerman *et al.* (1963) and Wilkinson (1966) have shown that all three major classes of immunoglobulin are involved at some time during the course of infection. The former workers showed that three purified immunoglobulins, known as IgG, IgA, and IgM, obtained from the serum of cases of brucellosis contained brucella agglutinating antibodies, and Wilkinson, using mainly cattle sera, found that when each of these immunoglobulins was removed by specific precipitating rabbit anti-human IgG, IgA, and IgM sera this resulted in the inhibition of their serological activity. Anderson *et al.* (1964) and Reddin *et al.* (1965) reported changes in serological activity during the course of the disease, and it seemed likely that analysis of a serum at different stages of the disease to determine in which fraction its serological activity lies might give information of value in diagnosis, as has been indicated in a previous study (Kerr *et al.*, 1966).

The present report is an attempt to relate the serological response at various stages of acute brucellosis to the types of globulin present at these stages, and to compare these findings with those found in sera from a group of veterinary surgeons who, while no longer acutely infected, may have been suffering from the disease in its chronic form or may have had their immune responses from a previous infection stimulated by repeated contact with the organism in the course of their work.

Source of Human Material

A series of 12 specimens of serum were examined from a case of acute brucellosis in a man aged 21, resulting from a laboratory infection (Case 1). The specimens were taken during a period of from 14 to 519 days after onset, five of them during the 35 days before the patient agreed to enter hospital for intensive antibiotic treatment.

A series of seven specimens of serum were examined from a case of acute brucellosis in a boy aged 14, probably resulting from the drinking of milk from an infected herd (Case 2). Five of the specimens were taken at weekly intervals, from 14 to 42 days after onset; all except the first one after antibiotic treatment had begun, and the seventh just over one year after his discharge.

Sera from 10 veterinary practitioners with serological evidence of contact with brucella organisms were examined. Three of them were from men who stated that they had suffered from acute brucellosis with periodic relapses. Three had had prolonged illness with symptoms suggestive of but not proved to be brucellosis, two had severe local and generalized reactions to accidental injection of the live brucella vaccine S19 while immunizing cattle, and two said that they had had no symptoms (see Table V).

Specimens of plasma from two blood donors which were found to contain agglutinating antibodies were examined. These were two of several thousand investigated of which approximately 1.5% were found to be positive in the standard agglutination test.

Methods

Agglutination Test.—This was carried out by the standard technique recommended by the Standards Laboratory of the Public Health Laboratory Service (P.H.L.S., 1961). Doubling dilutions of serum from 1/10 upwards in phenol-saline (0.5% phenol in normal saline) were tested in Dreyer's round-bottomed agglutination tubes (3 by ¼ in.; 7.5 by 0.6 cm.). The antigens used included the standard *Brucella abortus* and *Br. melitensis* (P.H.L.S.) antigens and the W.H.O. standard (Weybridge) suspension diluted 1/10. The tubes were placed in a water-bath at 37° C. and readings made after 48 hours. The titre was taken as the highest dilution of serum resulting in 25% clumping and some degree of clearing of the suspension.

Treatment with Mercaptoethanol.—The method of treatment has been described by Anderson *et al.* (1964). Agglutination tests were carried out with phosphate-buffered saline of *p*H 7, containing 0.05 M mercaptoethanol instead of normal phenol-saline as diluent. For comparative purposes agglutination tests were set up with and without mercaptoethanol.

Complement-fixation Test.—The technique of the test was similar to that of Bradstreet and Taylor (1962) with the use of W.H.O. plastic plates and standard drops of 0.1 ml. of serum, complement, antigen, and sensitized red cells respectively. As an antigen the P.H.L.S. *Br. abortus* antigen (as used in the agglutination test) was employed at an optimum dilution determined by chessboard titration. The tests were carried out in duplicate for greater accuracy, one being given short-period fixation at 37° C. for 20 minutes and the other long fixation by holding overnight at 4° C. Doses of complement of 1.2 $M.H.D._{100}$ were used in the short method and 2 $M.H.D._{100}$ in the long method. The titre was taken as the dilution of serum resulting in 50% fixation.

Serum Fractionation.—Sucrose density gradient fractionation of serum was carried out by the method of Kunkel (1960) and modified as described by Weir *et al.* (1966) for use in an M.S.E. Superspeed 40 T.C. centrifuge. The specimen of serum was first diluted 1/2 and 0.5 ml., then layered on to a gradient prepared with 1-ml. volumes of 40, 30, 20, and 10% sucrose in phosphate-buffered saline (*p*H 7.2). Fractionation was carried out by centrifugation for 16 hours at 35,000 r.p.m. Nine fractions of 0.5 ml. were then removed, starting from the top of the column. Each fraction was then tested for its serological activity by means of agglutination and complement-fixation tests.

Quantitative Immunodiffusion Test.—The quantitative immunodiffusion technique of Darcy (1960) was used to determine the relative amounts of the individual immunoglobulins in the sucrose density gradient fractions. Specific goat antisera to human IgG, IgA, and IgM obtained from Hyland Laboratories was used. The antisera were checked for specificity and showed reactions of non-identity in immunodiffusion tests. Each test was set up in duplicate and incubated at 37° C. for 72 hours ± 15 minutes and the position of the precipitin bands measured with a Maxta viewer.

Results

Sucrose density gradient fractionation of serum separates the immunoglobulins into two peaks of differing serological activity. In the system used here the low molecular weight (IgG and IgA) globulins are concentrated in fractions 3 and 4 and the high molecular weight (IgM) globulins concentrated in fractions 6 and 7. The positions of these immunoglobulins have been confirmed in this system by using dye-labelled IgG and IgM (Stanworth, 1967). Further confirmation was given by the fact that the IgM "Paul–Bunnell" antibody in a human serum from a case of glandular fever was found to be concentrated in fractions 6 and 7. There is, however, inevitably some slight overlapping of the different classes of immunoglobulins, and

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the quantitative immunodiffusion test (Darcy, 1960) with the use of Hyland antisera specific for the different immunoglobulin classes, while clearly showing the two peaks, reveals also some traces of IgA and IgG globulin in all fractions as far down as 6 and 7.

Table I shows the results of serological studies on the whole serum of a case of acute brucellosis taken two weeks after the onset of the disease and at various intervals thereafter until over one year later. It can be seen clearly that both the standard agglutination and the complement-fixation titres rose until treatment was begun and then fell off gradually, with perhaps a slight lag at first in the decrease of the complement-fixation titre, which had almost disappeared by the end of one year, leaving an agglutination titre of 1/640. The agglutination titre can also be seen to be markedly reduced by mercaptoethanol treatment known to affect primarily IgM-type and IgA (reaginic) globulins, so that the figures expressed in the third column reflect the level of the low molecular weight globulins (IgG) at each stage of the illness. In this case these antibodies fell rapidly shortly after the beginning of treatment and continued to decrease in parallel with the standard agglutination and the complement-fixation titres, until, like the latter, they reached a low level (1/80) at the end of one year.

Table II shows the results of fractionation studies on five of the serum samples shown in Table I—those taken at 14, 35, 56, 92, and 359 days after onset. It can be seen that there are two peaks of activity, one of which is associated with fractions known to contain the IgG and IgA—namely, fractions 3 and 4—and the other peak is associated with fractions known to contain IgM—namely, fractions 6 and 7. These peaks of activity are associated with both agglutination and complement fixation and it is seen that mercaptoethanol, as would be expected, has little or no effect on the agglutination activity associated with the IgG peak but markedly affects the activity associated with the higher molecular weight protein. Neither of the peaks predominate at any stage of the disease studied and they fall off together after treatment. Further, it is not possible to distinguish between the two peaks on the basis of their serological activity during the acute phase of the disease.

Table III shows the serological results of a series of serum specimens from Case 2. The first of these was obtained shortly after the patient's admission to hospital 14 days after the onset of symptoms and the second specimen one week later, after antibiotic therapy had begun. The last specimen was taken about 15 months after onset. The antibiotic treatment, started earlier than in the previous case, appears to have stopped the rise in the antibody levels, which remained more or less static up to 42 days after onset. A year later they were much reduced and appeared to be mainly of the low molecular weight type unaffected by mercaptoethanol.

Table IV shows that after fractionation of the serum samples both high and low molecular weight globulins were present and took part in the serological reactions. However, the results

TABLE I.—*Serological Reactions of Specimens of Serum at Different Stages During an Acute Attack of Human Brucellosis (Case 1)*

Days After Onset	Agglutination of *Br. abortus*		Complement Fixation
	Standard Test	In Presence of Mercaptoethanol	
14	3,840	120	64
20	> 15,360	480	256
25	> 15,360	480	256
33	61,440	3,840	512
35	122,880	7,680	256
	Intensive antibiotic treatment begun		
41	15,360	960	512
48	3,840	960	1,024
56	3,840	960	64
92	3,840	480	64
125	1,920	240	32
359	640	80	4
519	320	20	4

TABLE III.—*Serological Reactions of Specimens of Serum at Different Stages of Case 2. Results Expressed as Reciprocals of the Titres*

Days After Onset	Agglutination of *Br. abortus*		Complement Fixation
	Standard Test	In Presence of Mercaptoethanol	
14	640	640	> 128
	Antibiotic treatment begun		
21	1,280	640	> 128
28	1,280	320	512
35	1,280	640	512
42	1,280	320	512
54	320	160	64
443	80	40	8

TABLE II.—*Standard Agglutination, Agglutination in the Presence of Mercaptoethanol (M.E.), and Complement-fixation Titres in Tests Carried Out on Nine Fractions of Serum taken at different Stages of an Acute Brucella Infection (Case 1). (Titres are Expressed as Reciprocals)*

Fractions	Specimen 1 (14 days*)			Specimen 5 (35 days)			Specimen 8 (56 days)			Specimen 9 (92 days)			Specimen 11 (359 days)		
	Agglutination		C.F.	Agglutination		C.F.	Agglutination		C.F.	Agglutination		C.F.	Agglutination		C.F.
	Standard Test	M.E.		Standard Test	M.E.		Standard Test	M.E.		Standard Test	M.E.		Standard Test	M.E.	
1	< 60	·	·	< 60	·	·	< 60	·	·	< 60	·	< 4	< 10	< 10	< 4
2	< 60	·	< 4	20	10	4	40	< 10	8	< 60	·	< 4	< 10	< 10	< 4
3	20	10	8	240	480	64	120	240	32	120	·	8	< 10	10	8
4	40	20	8	240	480	32	60	240	32	120	·	16	< 10	< 10	4
5	40	20	< 4	40	40	8	20	40	8	60	·	8	10	< 10	Tr. 4
6	1,920	60	32	> 1,920	120	64	960	20	32	240	·	< 4	40	10	4
7	480	20	4	240	60	32	480	20	Tr. 4	480	·	< 4	10	10	Tr. 4
8	60	< 60	< 4	60	Tr. 10	< 4	60	< 10	< 4	120	·	< 4	< 10	< 10	< 4
9	< 60	< 60	·	60	< 10	< 4	< 60	·	·	< 60	·	< 4	< 10	< 10	< 4

* Days after onset. · Test not done.

TABLE IV.—*Standard Agglutination, Agglutination in the Presence of Mercaptoethanol (M.E.), and Complement-fixation Titres in Tests Carried Out on Nine Fractions of Serum taken at different Stages of an Acute Brucella Infection (Case 2) (Titres Expressed as the Reciprocal)*

Fractions	Specimen 2 (21 days)			Specimen 4 (30 days)			Specimen 5 (42 days)			Specimen 6 (54 days)		
	Agglutination		C.F.	Agglutination		C.F.	Agglutination		C.F.	Agglutination		C.F.
	Standard Test	M.E.		Standard Test	M.E.		Standard Test	M.E.		Standard Test	M.E.	
1	< 10	·	< 4	< 10	< 10	< 4	< 10	< 10	< 4	< 10	·	< 4
2	60	60	8	60	120	32	< 10	< 10	< 4	< 10	·	< 4
3	240	240	64	240	240	128	240	480	128	120	·	8
4	240	240	64	120	240	64	240	480	64	240	·	16
5	120	20	8	< 60	20	32	60	< 10	< 4	120	·	8
6	240	10	16	120	10	16	60	10	8	60	·	Tr. 4
7	60	10	8	60	< 10	16	< 10	< 10	4	< 10	·	< 4
8	40	< 10	< 4	< 10	< 10	A.C.	< 10	< 10	A.C.	< 10	·	< 4
9	< 60	< 10	< 4	< 60	·	< 4	< 10	< 10	A.C.	< 10	·	< 4

A.C. = Anticomplementary. · Test not done.

TABLE V.—*Serological Reactions of Whole Serum and Fractionated Serum from 10 Veterinary Practitioners*

	Previous History	Activity of Whole Serum: Agglutination: Standard Test	Activity of Whole Serum: Agglutination: M.E.	Activity of Whole Serum: Anti-human Globulin Test	Activity of Whole Serum: C.F.	Activity in Fractions 1–9: Standard Agglutination Positive Fractions	Activity in Fractions 1–9: Anti-human Globulin Test Positive Fractions	Activity in Fractions 1–9: C.F. Positive Fractions
1	Brucellosis with relapses	20	.	+	+	3, 4	2, 3, 4, 5	**2, 3, 4, 5**
2	" " "	80 (PZ)	80	+	+	(2), 3, 4, 5	2, 3, 4, 5	3, 4, 5
3	Symptoms suggestive of brucellosis	—	.	+	+	None	2, 3, 4, 5	2, 3, 4, 5
4	" " "	40 (PZ)	40	+	+	"	2, 3, 4, 5	2, 3, 4
5	Reaction to accidental injection of live vaccine	—	.	+	+	"	3, 4	2, 3, 4, 5
6	Symptoms suggestive of brucellosis	—	.	+	+	"	None	1, 2, 3
7	Reaction to accidental injection of live vaccine	—	.	+	+	"	2, 3, 4, 5, (6)	2, 3, 4, 5, (6)
8	No symptoms	—	.	+	+	"	2, 3, 4, 5	2, 3, 4, (5)
9	Brucellosis with relapses	> 320 (PZ)	320 (PZ)	+	+	2, 3, 4, 5, 6	2, 3, 4, 5, 6	2, 3, 4, 5, (6)
10	No symptoms	80 (PZ)	—	+	+	None	None	2, 3, 4

— = Negative at 1/10. ( ) = Trace reaction. PZ = Prozone. The fraction with the strongest reaction in each test is in bold type.

indicate that the high molecular weight globulins (IgM) were tending to decrease in the later specimens while the low molecular weight globulins (IgG) were still active.

Table V shows the results of serological investigation of 10 veterinary practitioners whose work brought them into contact with cattle. In the standard agglutination test one of the sera agglutinated *Br. abortus* strongly at 1/320 but had a prozone in dilutions up to 1/80. All the other nine sera failed to agglutinate *Br. abortus* or gave an indeterminate reaction with a prozone. They were, however, strongly positive in the anti-human globulin (Coombs) test. All 10 sera fixed complement.

The sera were submitted to sucrose density gradient fractionation and the nine fractions in each case were tested by standard agglutination, anti-human globulin (Coombs), and complement-fixation tests. The results showed that the serological activity was mainly confined to the low molecular weight (IgG and/or IgA type) immunoglobulins. Trace reactions were occasionally found in fraction 6 and may have been due to traces of IgM or to carry-over of IgG, but there was no evidence of marked activity by IgM, except perhaps in serum 9, consistent with the reduction in titre by mercaptoethanol. The peak of activity in every case was found in fraction 3 or 4. The absence of IgM antibodies was further confirmed by the fact that in those cases where standard agglutination by the whole serum was positive mercaptoethanol did not affect the result. It seems that the sera of veterinary surgeons are likely to contain antibodies of IgG or IgA type and little or no IgM antibodies.

The results of the serological investigation of plasma specimens from two blood donors are shown in Table VI. They show that agglutination in the standard test was due solely to antibodies that were sensitive to mercaptoethanol. The anti-human globulin (Coombs) test failed to show any nonagglutinating antibodies. The fact that they are high molecular weight immunoglobulins (IgM) was confirmed by fractionation. There was no evidence of serological activity in the early fractions where low molecular weight antibodies would be sited; but some degree of activity was detected in fractions 6 and 7.

Discussion

This investigation has shown that it is possible to distinguish two groups of individuals: (1) those acutely infected who have positive serological tests of all types due to activity associated with both low and high molecular weight immunoglobulins and who, if properly treated early on, show a marked reduction of and steady fall in the level of all types of antibody during a period which may last as long as 12 months or more; and (2) individuals who have either chronic brucellosis or whose immunity is being repeatedly stimulated by contact with brucella organisms. These latter give weakly positive or negative direct agglutination, whereas the complement-fixation and the anti-human globulin (Coombs) test are markedly positive. The serological activity in the second group was found to be associated with low molecular weight immunoglobulin and little or no high molecular immunoglobulin could be detected.

TABLE VI.—*Serological Reactions of Two Plasma Specimens and Their Fractions from Two Blood Donors*

Specimen	Agglutination: Standard	Agglutination: In presence of mercaptoethanol	Anti-human Globulin Test (for non-agglutinating antibodies)	Complement-fixation Test	Fractions Showing Serological Activity: Agglutination	Fractions Showing Serological Activity: Anti-human Globulin Test	Fractions Showing Serological Activity: C.F.
B.D. plasma 1	160	Negative	Negative	Negative	6 and 7	6 and 7	6
" " 2	320	"	Negative	Anti-complementary	6	6 and 7	6

The cured acute cases with their falling antibody levels follow the pattern of response expected after primary immunization and end up with low levels of antibody. The weakly positive sera occasionally found in the normal population—for example, blood transfusion specimens—probably fall into this category, the individuals concerned having had an unrecognized or inapparent brucella infection. This group contrasts with the group chronically infected or repeatedly immunized through contact (the veterinary surgeons), which shows a response typical of a booster or secondary response where the antibody is largely of the low molecular weight type as has been found after booster T.A.B. immunization (LoSpalluto *et al.*, 1962) with respect to the typhoid H antigen.

Summary

The serological findings with whole serum and sucrose density gradient fractions are given for two cases of acute brucellosis which were recognized and treated. Their serology was followed for more than 12 months from the onset of symptoms. The results are compared with similar studies on the sera of 10 individuals who may be suffering from the disease in its chronic form. In the latter cases the serum reactivity is mainly confined to the low molecular weight immunoglobulins (IgG and IgA) while in the acute cases high molecular weight (IgM) globulins are also involved.

The serological differences found between the two groups may prove valuable in distinguishing between acute and chronic forms of the disease.

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