INCOMPLETE ANTIBODIES IN BRUCELLOSIS: AN INDIRECT ANTIGLOBULIN (COOMBS) ONE-TUBE SCREEN TEST

A. ZOUTENDYK, M.R.C.S., L.R.C.P.

Blood Group Research and Transfusion Laboratories, The South African Institute for Medical Research, Johannesburg

Since the demonstration independently in 1944 by Race in Britain and Wiener in the USA of the existence of 'incomplete' and 'blocking' antibodies in connexion with rhesus sensitization and haemolytic disease of the newborn, increasing practical use has been made by immuno-haematologists of the fact that agglutinating antibodies may be divided into at least 2 and probably 3 main classes. One class manifests itself by agglutinating cells suspended in saline, the others require that the cells be suspended in a medium such as albumin or that they be treated by enzymes such as trypsin,

papain or ficin. In addition, a very delicate and possibly the most valuable and widely used technique for demonstrating incomplete antibodies is the indirect antiglobulin (Coombs) technique. If, therefore, in attempting to diagnose any given condition, whether haematological or bacteriological, one depends only on the classical saline agglutination reaction a large proportion of cases will inevitably be missed. Inexplicably, these fundamental differences in antibodies have not been utilized to any extent by immuno-bacteriologists, although studies on these lines have been carried out by a number of research workers in many parts of the world during the past twelve years. Morgan and Schutze[1] applied the Coombs technique to the detection of shiga and typhoid antibodies and in many cases found considerably higher titres than by the conventional agglutination techniques. The results were confirmed and the work extended by Stewart and McKeever.[2] Griffiths[3] was the first to demonstrate the blocking mechanism in brucellosis by the use of albumin or serum and obtained positive results when saline tests were negative. Coombs and Stoker[4] investigated the method in the diagnosis of Q fever and concluded that the antiglobulin test was much more sensitive than direct agglutination and promised to be specific. For brucellosis Wilson and Merrifield[5] studied 130 sera from cases of 'PUO' and suspected brucellosis, the antiglobulin technique revealing significant titres where conventional methods were negative. The prozone phenomenon, so troublesome in brucellosis, was eliminated and they decided that the method could therefore be used as a single-tube screening test in the diagnosis of the disease. Wagner and Kuhns[6] found that the modified Coombs test appeared to possess greater specificity and sensitivity than the usual agglutination reactions. They considered that the method might be of value in establishing previous exposure to brucella organisms when other methods fail. In South Africa Coetzee[7] has investigated 400 hospital patients thought not to be suffering from brucellosis, 50 abattoir workers and 17 Onderstepoort laboratory workers, and on the strength of his findings has recommended a one-tube anti-globulin screening test, at a dilution of 1/20 or 1/40 by his technique, in the diagnosis of brucellosis. He considers it to be sensitive and specific.

The present long-term investigation into the diagnosis of brucellosis had its origin in our researches into the haemolytic anaemias; during the past 10 years we have, wherever practicable, screened our patients for a possible infective aetiology for the haemolytic process and in this connexion brucellosis has always been borne in mind. It was felt that, in the light of advances in the knowledge of antibodies, the classical saline agglutination technique was not sufficient to exclude past or present brucella infection and a beginning was made to apply the indirect Coombs reaction for brucellosis to our cases of acquired haemolytic anaemia. It was soon appreciated by colleagues that the test had a much wider clinical application and during the past 3 years more than 2,000 investigations have been carried out. In an attempt to assess the practical utility of the test, the patients have been classified according to the provisional diagnosis provided by the clinicians. The 'no diagnosis given' category which, unfortunately, is always a disappointingly large group in every investigation in clinical pathology, nevertheless serves as a useful control group of miscellaneous patients. Three hundred specimens of blood were taken at random from European and African ante-natal patients and blood donors to serve as a control of the general population.

TECHNIQUE

From the outset the investigation was designed as a one-tube screen test, partly for practical reasons, because the technique is somewhat laborious, and partly because titrations based on agglutination reactions are relatively inaccurate and therefore potentially misleading. The problem of the optimum serum dilution to be used was difficult to decide; various workers have suggested dilutions varying from 1/5 to 1/40 but generally the reactions have been carried out at high temperatures such as 50°C and for incubation times as long as 24-48 hours. After many preliminary investigations it was decided to adopt a final dilution of 1/5 for the screen test because the control group showed that the proportion of the random population giving a positive reaction by the technique used was no larger than would be expected from a condition as prevalent as brucellosis. A negative reaction at the dilution of 1/5 would be reasonably certain to exclude past or present brucella infection whilst higher dilutions or a complete titration can be carried out where indicated.

In the preliminary stages, suspensions of both *Brucella melitensis* and *Br. abortus* were used in parallel but it was found that this was an unnecessary refinement and the antigen finally decided upon was a heat-killed suspension of *Brucella abortus* strain 19 used at opacity 4. Throughout this work the antigen has been kindly supplied by Dr. V. Bokkenheuser, of the Institute's Serological Division, and is identical with the antigen used in his department for the saline agglutination test for brucellosis, the results being thus comparable; in fact in the early stages a considerable amount of comparative work was carried out in both departments.

The patient's serum should be spun completely free of red cells, after which 4 drops of the serum are mixed with 16 drops of the diluted antigen, the mixture being placed in a water bath at 37°C for 3 hours. The tubes are then spun for 1 minute and are examined for the presence of saline agglutinins. If, as is generally the case, no saline agglutinins are demonstrable, the mixture is washed twice in 5 ml. of saline by spinning for 2 periods of 15 minutes. After the second washing the saline is pipetted off carefully and 12 drops of saline are added; the button of packed organisms is broken up thoroughly and the suspension is distributed into 3 tubes of 4 drops each. Two dilutions of anti-human-globulin serum are used, the optimum dilutions for each batch being decided upon by titrating known positive reacting sera from patients. The majority of anti-human sera used for this purpose have reacted satisfactorily at dilution of 1/30 and 1/60 and in practice 4 drops of the 1/30 dilution are added to the front tube, 4 drops of the 1/60 dilution to the middle tube, and 4 drops of saline to the 3rd tube, this acting as a saline control. The tubes are shaken and placed in the water bath at 37°C. They are examined macroscopically or, if necessary, by the aid of a hand lens and as the reaction is in the nature of a flocculation the tubes should be inspected at regular intervals for the first appearance of the floccules. The entire test is completed in one day; the mixtures are made at 9 a.m. and they are washed from 12.15 p.m. to 12.45 p.m., after which the anti-human is added. The first positive signs are usually visible after 1 hour and the final result can be seen after a further 2 hours. No improvement has been found by leaving the tubes in the water-bath overnight although this is usually done for convenience and a final check. Controls are included with every batch of tests. Considerable experience is needed in the interpretation of the results, which we have found useful to classify as weak, moderate and strong; it is recommended that weak reactions be confirmed and compared at a later date.

RESULTS

The results obtained in 2,393 consecutive investigations are shown in Table I. It will be seen that 5.0% of the random population tested possess incomplete antibodies against brucellosis as compared with 20.6% of the patients in whom practitioners considered it necessary to confirm or exclude

TABLE I. RESULTS OF MODIFIED COOMBS TEST FOR BRUCELLOSIS OVER A PERIOD OF 3 YEARS

Provisional Diagnosis	*Europeans*		*Natives*		*Total*	
	Positive	*Negative*	*Positive*	*Negative*	*Positive*	*% Positive*
Arthritis	41	131	39	138	80	22·9
Acute rheumatism	1	34	3	21	4	7·0
Brucellosis	44	138	17	47	61	24·8
PUO	64	249	25	142	89	18·5
Backache	13	4	1	2	14	70·0
Pneumonitis	4	9	1	3	5	19·4
Cardiac	4	4	0	1	4	44·0
Anaemia	9	13	2	12	11	30·5
Adenitis	8	20	1	6	9	25·8
Hepatosplenomegaly	7	21	1	8	8	21·6
Intestinal	5	24	4	11	9	20·4
Ocular	4	2	—	—	4	—
Hepatitis	3	7	3	3	6	37·5
CNS	2	18	—	—	2	10·0
Bone	4	15	4	5	8	28·5
Tuberculosis	6	3	0	2	6	54·5
No diagnosis given	94	535	24	60	118	16·5
Totals	313	1,224	125	461	438	20·6
Random controls	4	146	11	139	15	5·0

brucella infection. The positive rate among random European controls is 2.6% as compared with 7.3% among random Natives and the value of a positive result in the latter is correspondingly reduced. Figures relating to some of the individual provisional diagnoses are worthy of comment:

'*Brucellosis*.' It is not sufficiently appreciated that a provisional diagnosis such as brucellosis or, worse still, brucellosis, conveys no information to the clinical pathologist; reference to one or more symptoms, signs or systems would be much more informative. However, where this provisional diagnosis was given about 25% of the sera gave a positive screen test.

'*Arthritis*.' Over 23% of the investigations were positive and frequently the difficult problem arises whether the condition is rheumatoid arthritis or an arthritic manifestation of brucellosis. An important practical point is the advisability of carrying out both a Coombs test for brucellosis and a Rose test for rheumatoid arthritis. The results obtained in 293 cases where both tests were asked for is shown in Table II. It is generally conceded that the differential sheep-cell

TABLE II. COMPARATIVE RESULTS OF THE ROSE TEST AND MODIFIED COOMBS TEST IN 'ARTHRITIS'

Rose Neg. Coombs Pos.	*Rose Pos. Coombs Neg.*	*Rose Pos. Coombs Pos.*	*Rose Neg. Coombs Neg.*
58	27	5	203

agglutination test for rheumatoid arthritis has a high degree of specificity and will detect about 80% of cases. It is particularly interesting therefore that in only 5 instances were the Rose test and the modified Coombs test both positive in the same patient. It is important to distinguish rheumatoid arthritis from brucellosis in children but it is unfortunate that in young patients the Rose test is often negative, even in Still's disease. Probably the best practical approach to the problem is appreciation of the fact that rheumatoid arthritis is relatively uncommon in children and, contrary to the usual belief, that brucellosis is more common than is generally supposed. It has been calculated (Gauchat and May[8]) that 1,200 (3 per 100,000) new cases of rheumatoid arthritis occur per year in the American child population and, if a similar frequency holds good for South Africa, one would not expect to see more than about 30 new cases per year in the European under-15 child population of the Union. It is interesting that, although the numbers are not large, the percentage of cases of acute rheumatism giving a positive Coombs test for brucellosis is not significantly different from that in the random population.

'*Backache*.' Almost three-quarters of the cases of backache gave a positive reaction; this result, surprising at first sight, fits in with the liability of brucellosis to involve bones and joints. Hodgkinson[9] in describing a case of brucellosis of the lumbar spine emphasizes that brucellosis as a cause of backache should always be considered. Emik *et al*[10] in a valuable analysis of brucella patients in Indiana over a 5-year period describe a 5-symptom complex in which backache was noted in over 55%—about the same as night sweats and exceeded in frequency only by weakness (the most prominent symptom), evening rise in temperature, chills and headache. Arthritis was present in about 18% of the patients.

'*PUO*.' This diagnosis, with in many cases a history of sweats, weakness and vague ill-health accounted for over 18% positives. The interpretation of the result would depend to a great extent on the exact history and the combination and duration of the accompanying symptoms and signs.

'*Miscellaneous*.' The numbers in the miscellaneous group are not sufficiently large to warrant firm conclusions being drawn about any individual condition. However, the wide scope of the list is a tribute to the protean nature of the manifestations of brucella infection. Of particular interest are the ocular, pulmonary and hepatic conditions in which antibodies were demonstrable. An important finding was the positive result in 5 cases of suspected tuberculosis; in this connexion it should be remembered that brucellosis can give rise to lesions difficult to distinguish from tuberculosis and in one recorded case a kidney was removed before the brucella aetiology became apparent (Abernethy *et al*.[11]). In this connexion an interesting Rhodesian case of chronic brucella pyelonephritis with calcification has recently been described by Honey, Gelfand and Myers [12] and they also stressed the close similarity between renal brucellosis and renal tuberculosis.

DISCUSSION

Brucellosis, especially the chronic form, is not a clinical diagnosis; the clinician requires laboratory confirmation and it will be generally conceded that with existing tests such confirmation is rarely forthcoming. Consequently, as stated previously, brucellosis is often considered to be an uncommon condition, especially in children, whereas in fact it is very common, being endemic in some parts of South Africa and a cause of much chronic ill-health (S.A.I.M.R. Annual Report, 1956, p. 43). Wallis[13] in his paper on brucellosis in children has summarized the problem very aptly as follows: 'Brucellosis is a disease of mistakes. If we do not think of it, we miss it; or we may think of it and test for it, yet find nothing. It eludes us.' There must be some explanation for

this elusiveness and it is logical to assume *inter alia* that the existing techniques are deficient since they take no cognisance of incomplete antibodies and therefore fail to detect a considerable proportion of cases. Positive blood cultures are disappointingly infrequent and in any case may need weeks before an opinion can be given. Positive skin tests show that the patient has been exposed to brucella infection and their chief value probably lies in the diagnostic significance of the appearance of agglutinins or a rise in agglutinin titre after a skin test has been carried out. The classical saline agglutination reaction is also of limited usefulness; after half a century there is still no unanimity of opinion about temperature, time and other technical details, whilst the attempt to define a so-called 'diagnostic' titre is obviously fallacious. No claim for any considerable degree of accuracy can be made for routine agglutination reactions, so that anything less than a spectacular difference in titre means little. More important, however, is the fact that the saline agglutination reaction will not detect the incomplete antibodies and the two tests should be complementary to one another, as they are, for example, in the investigation of rhesus sensitization. The usual saline agglutination test should be done and if this is negative, the antiglobulin reaction should be carried out on the same mixture. Whether one uses the one-tube technique described in this paper or a full-scale titration or, possibly, albumin or enzyme techniques, will depend on personal preference and future research. There can be no doubt, however, that incomplete antibodies should be tested for not only in suspected brucellosis but in other conditions where agglutinins are relied on as an aid to diagnosis.

One is constantly being asked about the degree of specificity of the antiglobulin test for brucellosis; no categorical answer can be given because few if any laboratory tests depending on an antigen-antibody reaction can be specific for any one disease. In the light of the information afforded by the present investigation one can say that, with the technique employed, about 5% of the random population will give a positive indirect Coombs test for brucellosis, although the figure for Europeans is less than 3% and for Natives over 7%. This means that they have been exposed either to brucellosis or an antigen of a similar structure; it is known, for example, that immunization with cholera vaccine may lead to the production of agglutinins against brucella. The results obtained suggest that there may be some cross action with tuberculosis and in one case a strongly positvie brucella Coombs reaction was obtained in a patient in whom subsequently a positive blood culture for typhoid was obtained (Dr. I. J. Grek, personal communication).

The actual interpretation to be placed on a positive modified Coombs test differs in no respect from the interpretation to be placed on a saline agglutinin result, a positive skin test, or any other investigation short of a positive blood culture. It means that the patient has, or has had, brucellosis, which a negative result would almost certainly exclude. This makes it as valuable in brucellosis as the TPI test is in syphilis; the ultimate decision whether the disease is active or not must be made by the clinician. The evaluation of any laboratory test depends on its clinical usefulness and whether it affords information which has hitherto been lacking. These questions can only be answered by the clinicians, and it is hoped that our results collected during the past 3 years will help to stimulate interest and further research into the role of incomplete antibodies in bacterial diseases.

SUMMARY

A one-tube screen test for incomplete brucellar antibodies is described.

The results obtained in over 2,000 consecutive tests are classified according to the provisional diagnosis.

A plea is made for further research into incomplete antibodies in all bacterial conditions in which saline agglutination tests are at present carried out.

REFERENCES

1. Morgan, W. J. J. and Schutze (1946): Brit. J. Exp. Path., **27**, 286.
2. Stewart, F. S. and McKeever, J. D. (1950): J. Hyg. (Camb.), **48**, 357.
3. Griffitts, J. J. (1947): Publ. Hlth. Rep. (Wash.), **62**, 865.
4. Coombs, R. R. A. and Stoker, M. G. P. (1951): Lancet, **2**, 15.
5. Wilson, M. M. and Merrifield, E. V. O. (1951): Lancet, **2**, 913.
6. Wagner, B. M. and Kuhns, D. M. (1953): Amer. J. Clin. Path., **23**, 185.
7. Coetzee. J. N. (1956): S. Afr. J. Lab. Clin. Med., **2**, 259.
8. Gauchat, R. D. and May, C. D. (1957): Pediatrics, **19**, 672.
9. Hodgkinson, R. (1955): Med. J. Austral., **2**, 205.
10. Emik, L. O., Damon, S. R. and Scruggs, J. H. (1957): Publ. Hlth. Rep. (Wash.), **72**, 655.
11. Abernethy, R. S., Price, W. E. and Spink, W. W. (1955): J. Amer. Med. Assoc., **159**, 1,534.
12. Honey, R. M., Gelfand, M. and Myers, N. H. (1957): Cent. Afr. J. Med., **3**, 465.
13. Wallis, H. R. E. (1957): Brit. Med. J., **1**, 617.