AN OX CELL HAEMOLYSIN TEST FOR THE DIAGNOSIS OF INFECTIOUS MONONUCLEOSIS

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I. INTRODUCTION

While the majority of overt cases of infectious mononucleosis can be diagnosed on clinical grounds alone, it is widely recognized that this disease may assume widely varying forms. Routine investigation for infectious mononucleosis of many apparently unrelated clinical conditions may frequently result in the demonstration of a common aetiology, and may even modify the accepted views of the prognosis of the disease. Haematological investigation is not a certain method of confirming the diagnosis. The cellular response is variable and may be atypical (Foord & Butt, 1939; Kruger, Wallace & Penchansky, 1945); the classical mononuclear cells may be found in other conditions (Warren, 1941; Randolph & Gibson, 1944), and the differentiation of the cells from those of acute lymphoblastic leukaemia may be impossible.

The diagnosis of the disease is greatly aided by the demonstration of the antibody response first shown by Paul & Bunnell (1932). The original test for sheep cell agglutinins, together with the major modifications (Davidsohn, 1937; Barrett, 1941), suffers from three limitations. First, not all cases of infectious mononucleosis give a positive test. Secondly, an arbitrary dilution has to be selected by the individual worker as an index of abnormality. Finally, either a considerable time of incubation, or in default of this, centrifugalization is necessary for the test and confirmatory absorption. The multiplicity of manoeuvres is likely to deter both clinician and pathologist from undertaking the large number of tests which the varied symptomatology of infectious mononucleosis warrants. Many techniques have been described which overcome these objections in part, but none is entirely satisfactory.

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Despite the fact that Davidsohn & Walker (1935) and Stuart, Fulton, Ash & Gregory (1936) had shown that the antibodies in infectious mononucleosis differed from the classical Forssmann type, the role of ox cells in the serological diagnosis was relegated to that of confirmation. Bailey & Raffel (1935) and Stuart, Griffin, Wheeler & Battey (1936) demonstrated that the haemolytic titre to ox cells was greatly raised while the agglutinin titre was scarcely affected. Gleeson-White, Heard, Mynors & Coombs (1950) confirmed this finding, and showed that, whereas the cells of individual oxen reacted variably to agglutination, all could be haemolysed to the same degree. Beer (1936) and Foord & Butt (1939) performed ox cell haemolysin tests on a few cases, generally confirming expected results.

No further references to the use of direct ox cell antibody estimations in infectious mononucleosis could be found, and it was considered that further investigation was desirable. The fact that infectious mononucleosis antibodies demonstrated by the use of sheep cells are absorbable by ox cells invites the use of the latter as the diagnostic antigen. If the demonstration of ox cell antibodies alone gave results equal to or better than those by existing techniques, the serological diagnosis could be simplified technically, and the number of serologically negative cases might be reduced. Since naturally occurring ox cell antibodies are normally absent, or present in very low concentration, and are not known to be raised in any conditions other than infectious mononucleosis and serum sickness, a single tube test could be devised which would permit the testing of large numbers of sera and which, it was hoped, would meet many of the objections to other diagnostic procedures. The primary purpose of this paper is to report preliminary results of the use of a one-tube ox cell haemolysin test in infectious mononucleosis.

II. MATERIALS AND METHODS

(*a*) *Complement*

Freeze-dried guinea-pig complement (Lyovac) has been used throughout. Three batches of complement were titrated for naturally occurring anti-ox cell antibodies. None of the samples caused haemolysis of ox cells at a dilution of 1:8 or weaker. Such antibodies could not have influenced the experiment since complement was never used stronger than 1:18. Absorption of fresh guinea-pig serum by ox cells is impracticable as lysis occurs in some degree in spite of taking all precautions. Boiled ox cells are ineffective as absorbing agents.

The standardization of complement dosage using ox cells and infectious mononucleosis antibody would be desirable; however, if an excess of complement is present, the exact dose is immaterial within broad limits. We have found it convenient to use 3 M.H.D. of complement as recommended for Wassermann tests (Wyler, 1929).

(*b*) *Human sera*

These were inactivated at 56° C. for half an hour before use.

(*c*) *Sheep cells*

Defibrinated sheep blood preserved with 0·1% formaldehyde was obtained commercially each week and stored at +4° C. Before use the cells were washed

three times and diluted to 1% in saline. The method of Stuart, Burgess, Lawson & Wellman (1934) has been used to determine sheep cell agglutination, the results being read macroscopically. Titres are expressed as the dilution of serum before addition of cells.

(*d*) *Absorbing antigens*

25% boiled ox cells and 25% guinea-pig kidney emulsion were prepared and used as described by Davidsohn (1938).

(*e*) *Ox cells*

Ox blood was collected at the slaughter house in bottles containing glucose citrate and stored at +4° C. For use the cells were washed three times and diluted to 2% in saline. Cells which were more than 6 days old were not used.

(*f*) *Ox cell test*

One hundred normal sera were titrated for naturally occurring ox cell antibodies with the results shown in Table 1. A serum dilution of 1:6 was therefore selected for use in a routine test. The technique was deliberately based on that used for

Table 1. *Naturally occurring ox cell haemolysins in human sera*

					Total
Titre ...	Negative	1:2	1:4	1:8+	.
Number ...	94	5	1	.	100

Wassermann tests in this laboratory; 0·022 ml. of serum (1 drop from a 56 dropper pipette, Starrett gauge) was diluted with 0·11 ml. saline (2 drops from a 30 dropper pipette), 0·11 ml. of 2% ox cells were added and the mixture incubated at 37° C. for 15 min. 0·11 ml. complement was added and incubation prolonged for a further 30 min.; centrifugation was not used. For experimental purposes, sera showing a positive test were titrated, titres being expressed as the dilution of the serum before addition of cells and complement.

III. RESULTS

(*a*) *Normal controls and diseases other than infectious mononucleosis*

An essential preliminary, as emphasized by Barrett (1941), is the determination of the level of sheep cell agglutinins which can be accepted by the individual worker as normal. One hundred normal sera were titrated for sheep cell agglutinins. The results are shown in Table 2. It was apparent that a titre of 1:64

Table 2. *Sheep cell agglutinins in normal persons*

								Total
Titre ...	Negative	1:2	1:4	1:8	1:16	1:32	1:64+	.
Number of persons ...	37	31	17	11	3	1	0	100

would be highly suggestive of abnormality in the serum while a titre of 1:32 or less could not be called abnormal unless absorptions confirmed them as such.

The ox cell haemolysin tests was carried out on 100 normal sera, comprising

fifty-five specimens from hospital staff and forty-five sera submitted for Wassermann testing from patients with no evidence of disease—for example routine ante-natal tests. Of these only two were positive. One originated from a man who was later found to have had an iritis of short duration; the sheep cell agglutinin titre of this sample was 1:32 and the ox cell haemolysin titre 1:16. While this may have been an atypical case of infectious mononucleosis, no further investigation was possible. The other came from a healthy laboratory technician from whom repeated samples continued to show an ox cell titre of 1:8 compared with a sheep cell agglutinin titre of 1:4. These two false results in one hundred samples compare broadly with the 1·7% found by Barrett (1941) using his sheep cell-absorption technique.

Diamond & Sennott (1945) having reported enhanced sheep cell agglutinin titres in infants and children, ox cell tests were performed on twenty children under 6 years old, who were in hospital for miscellaneous reasons. All were negative, but a modified technique was used to avoid venepuncture; blood was diluted 1:11 in saline in a white cell counting pipette, the cells were spun off and unit volume of the supernatant was used in the test which therefore was undertaken at an approximate dilution of 1:20.

Specificity of the test was further evaluated by testing 200 sera from hospital patients with diseases other than infectious mononucleosis. So far as was possible patients were chosen who were suffering from conditions in which early infectious mononucleosis might be considered in the differential diagnosis. The diagnoses were as follows: pharyngitis 40, lymphadenopathy (localized and generalized) 37, pyrexia of uncertain origin 25, jaundice 20, rashes of varying aetiology 16, unexpected positive Wassermann test 12, nasopharyngitis 9, general ill health 9, atypical chest conditions 8, acute abdominal conditions 9, splenomegaly 3, neurological disorders 7, mumps 4, hydatid disease of the liver 1.

Of these only four were positive; three of these (cases 24, 25 and 26) are described in the Appendix and the fourth, with further discussion, is described on p. 477. It is of interest that ten of the 200 cases, all of which were examined for sheep cell agglutinins, showed unabsorbed antibody to a titre of 1:32 or over. The finding of an increased incidence of sheep cell antibodies in high titre in persons suffering from disease as compared with normal persons accords with the findings of Dempster (1946).

(*b*) *Infectious mononucleosis*

The result of testing sixty cases of infectious mononucleosis for sheep cell agglutinins and by the test for ox cell haemolysins are shown in Table 3. The seven cases in column 3 include two cases which were originally negative to both tests, but in which the ox cell test became diagnostic before that using sheep cells.

It will be seen that the two tests agree in detecting antibody in about 63% of cases diagnosed clinically as infectious mononucleosis and in failing to detect antibody in some 23% of cases. Eight cases, however, were at first positive by one test only, seven by the ox cell test and one by the sheep cell test. This last (case 23) is discussed in the Appendix.

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Some further details of the seven cases in which the ox cell test appeared to be more satisfactory than that using sheep cells are given in Table 4. The sheep cell titres in the first instance were in all the cases too low to permit of satisfactory absorption. Confirmatory absorptions were obtained in at least one later specimen in all except cases 19 (see Appendix) and 22.

Table 3. *Serological reactions at the first test of cases diagnosed clinically as infectious mononucleosis*

					Total
Sheep cell agglutinin ...	+	+	−	−	.
Ox cell haemolysin ...	+	−	+	−	.
	38 (63·3 %)	1 (1·7 %)	7 (11·7 %)	14 (23·3 %)	60

Table 4. *Seven cases of infectious mononucleosis in which the ox cell haemolysin proved superior to the sheep cell agglutinin test*

		In hospital			Convalescent		
Case 16	Sheep agglutinins	1:4	1:16	.	.	.	.
	Ox cell screening test	+	+	.	.	.	.
	Ox cell titre	1:8	1:128	.	.	.	.
Case 17	Sheep agglutinins	1:4	1:128	.	1:64	.	.
	Ox cell screening test	+	+	.	+	.	.
	Ox cell titre	1:8	1:256	.	1:8	.	.
Case 18	Sheep agglutinins	1:4	1:4	.	1:16	1:4	.
	Ox cell screening test	+	+	.	+	+	.
	Ox cell titre	1:16	1:32	.	1:32	1:8	.
Case 19	Sheep agglutinins	1:2	.	.	1:4	1:2	1:4
	Ox cell screening test	−	.	.	−	−	+
	Ox cell titre	−	.	.	−	−	1:8
Case 20	Sheep agglutinins	1:4	1:4	1:16	1:32	1:2	.
	Ox cell screening test	−	±	+	+	−	.
	Ox cell titre	−	1:4	1:8	1:32	−	.
Case 21	Sheep agglutinins	−	1:128	.	−	.	.
	Ox cell screening test	+	+	.	−	.	.
	Ox cell titre	1:8	1:128	.	−	.	.
Case 22	Sheep agglutinins	1:2	.	.	1:2	.	.
	Ox cell screening test	+	.	.	−	.	.
	Ox cell titre	1:8	.	.	−	.	.

Further serological results in fifteen of the cases of infectious mononucleosis are set out in Table 5. The tests listed for each case in Tables 4 and 5 appear in chronological order only. It should not be supposed that the time lapse between tests is the same, nor does it follow that all patients were in the same stage of the disease when first tested. It is not considered that a more full exposition of each case would be of value. The serum from all these cases gave satisfactory absorptions at the first test.

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Table 5. *Comparison of sheep cell agglutinins and ox cell haemolysins in fifteen cases of infectious mononucleosis tested two or more times*

		In hospital			Convalescent		
Case 1	Sheep cell agglutinins	1:128	.	.	1:512	1:32	.
	Ox cell screening test	+	.	.	+	+	.
	Ox cell titre	1:128	.	.	1:256	1:32	.
Case 2	Sheep cell agglutinins	1:128	.	.	1:16	.	.
	Ox cell screening test	+	.	.	−	.	.
	Ox cell titre	1:8	.	.	−	.	.
Case 3	Sheep cell agglutinins	1:8	1:16	.	1:16	.	.
	Ox cell screening test	+	+	.	−	.	.
	Ox cell titre	1:8	1:64	.	−	.	.
Case 4	Sheep cell agglutinins	1:64	1:256	1:32	1:16	1:4	.
	Ox cell screening test	+	+	−	−	−	.
	Ox cell titre	1:128	1:64	−	−	−	.
Case 5	Sheep cell agglutinins	1:512	.	.	1:64	1:32	.
	Ox cell screening test	+	.	.	+	−	.
	Ox cell titre	1:256	.	.	1:8	−	.
Case 6	Sheep cell agglutinins	1:16	.	.	1:16	.	.
	Ox cell screening test	+	.	.	−	.	.
	Ox cell titre	1:32	.	.	−	.	.
Case 7	Sheep cell agglutinins	1:1024	.	.	1:10240	1:256	.
	Ox cell screening test	+	.	.	+	+	.
	Ox cell titre	1:512	.	.	1:256	1:64	.
Case 8	Sheep cell agglutinins	1:128	.	.	1:256	1:32	.
	Ox cell screening test	+	.	.	+	+	.
	Ox cell titre	1:64	.	.	1:64	1:8	.
Case 9	Sheep cell agglutinins	1:128	.	.	1:64	1:64	−
	Ox cell screening test	+	.	.	+	+	−
	Ox cell titre	1:64	.	.	1:8	1:8	−
Case 10	Sheep cell agglutinins	1:64	.	.	1:64	.	.
	Ox cell screening test	+	.	.	+	.	.
	Ox cell titre	1:64	.	.	1:16	.	.
Case 11	Sheep cell agglutinins	1:64	.	.	1:16	1:8	1:2
	Ox cell screening test	+	.	.	+	−	−
	Ox cell titre	1:32	.	.	1:16	−	−
Case 12	Sheep cell agglutinins	1:128	.	.	1:8	.	.
	Ox cell screening test	+	.	.	+	.	.
	Ox cell titre	1:32	.	.	1:8	.	.
Case 13	Sheep cell agglutinins	1:64	1:64	.	1:32	1:16	.
	Ox cell screening test	+	+	.	+	−	.
	Ox cell titre	1:64	1:8	.	1:8	−	.
Case 14	Sheep cell agglutinins	1:64	1:128	.	1:8	.	.
	Ox cell screening test	+	+	.	−	.	.
	Ox cell titre	1:256	1:64	.	−	.	.
Case 15	Sheep cell agglutinins	1:64	1:128	.	1:32	1:2	.
	Ox cell screening test	+	+	.	+	−	.
	Ox cell titre	1:32	1:64	.	1:32	−	.

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IV. DISCUSSION

(*a*) *Relating to specificity*

It is generally agreed (Durupt, 1937; Barrett, 1941; Dempster, 1946; Sohier & Girier, 1946) that the serological diagnosis of infectious mononucleosis depends on the presence of sheep cell agglutinins which are not absorbable by guinea-pig kidney, positive absorption with ox cells being confirmatory only. Differentiation between the antibodies of infectious mononucleosis and serum sickness is the only reason why attention should be focused on the avidity of the immune bodies for guinea-pig tissue rather than ox cells. The cases reported in this paper contain no patient suffering from serum sickness, but there is every reason to suppose that the technique described would result in false positive tests, since Beer (1936) has shown that haemolysins for ox cells are present in this condition. However, this may not be a serious objection to the test. In the first place, serum sickness is now exceedingly rare. Secondly it is doubtful whether the sheep cell agglutinins present normally in serum sickness and in infectious mononucleosis can be differentiated with certainty by existing techniques. Indeed, Sohier & Girier (1946) found five out of 157 sera from cases of serum sickness (3·2%) to give the reactions of infectious mononucleosis. Conversely they also report five cases of infectious mononucleosis in which guinea-pig absorption was positive. A similar case has been seen in our laboratory (see Appendix, case 2). Absorption of sheep cell agglutinins by both guinea-pig tissue and ox cells is not uncommon, apart from serum sickness, and accounts for three of the four false positive ox cell haemolysin tests noted on p. 474.

The remaining false positive test (case 27) was unfortunately inadequately examined. A case of florid secondary syphilis showed sheep cell agglutinins to a titre of 1:16 and ox cell haemolysins to 1:8. No further examination of this serum was possible and no other samples were obtainable. False positive Wassermann reactions have been often reported in infectious mononucleosis (Davis, 1944), but increased ox cell haemolysins in syphilis have not been described. This case raised the possibility that reaction between the antibodies of infectious mononucleosis and the extract of ox cells, which must be present in Wassermann antigens prepared from ox heart, might account for such an atypical result. It was confirmed that an alcoholic extract of ox cells contained no hapten capable of reacting with the antibodies of infectious mononucleosis (Stuart, Griffin, Wheeler & Battey, 1936; Stuart, Griffin, Fulton & Anderson, 1936). Nevertheless, a very short series of cases showed definite evidence that the elinin fraction obtained from ox cells by the method of Carter (1949) was active in this respect. Further investigation of this phenomenon may prove interesting, though its significance in the interrelationship of the serological reactions of syphilis and infectious mononucleosis appears very doubtful.

(*b*) *Relating to sensitivity*

All published series of cases of infectious mononucleosis contain a proportion of cases serologically negative by the sheep cell agglutination technique. It was originally hoped that haemolysis of ox cells might prove more sensitive than the

agglutination of sheep cells sufficiently often to reduce significantly the number of serologically negative cases. The results in this respect are disappointing. Of the seven cases in Table 4 only two failed eventually to produce sheep cell agglutinins in sufficient concentration to permit of adequate absorptions. However, the results in Tables 4 and 5 show that, of the cases, twelve showed a steadily falling ox cell haemolysin titre while seven showed a rising sheep cell agglutinin titre. All the seven cases in Table 4 showed an abnormal ox cell haemolysin titre before sheep cell agglutinins of diagnostic value were obtained. It is possible that the ox cell antibodies tend to appear and to fall off earlier than those to sheep cells, which may account for the apparent lack of sensitivity to the ox cell test shown by case 23 (see Table 3).

All the sera from the fourteen persistently negative cases were titrated against sheep cells using concentrated bovine albumin as diluent (Diamond & Denton, 1945). No evidence of incomplete antibody was found and there was no enhancement of agglutinin titre, which conflicts with the results of Milzer & Nathan (1947). Following the findings of Coombs & Hole (1948) it was considered possible that a preference for a complement other than that of guinea-pig might, if demonstrated, reduce the number of serologically negative cases. A further study, to be communicated separately, was made of the utilization of various complements by an infectious mononucleosis/ox cell system. Preliminary results show that guinea-pig, horse, pig, human and cat complements are fixed nearly equally by ox cells in the presence of infectious mononucleosis antibody. The fixation of pig and cat complements by human antibody in this system is of particular interest.

(c) *Practical application*

It is believed that the demonstration by a routine test of ox cell haemolysins in infectious mononucleosis gives results which do not suffer by comparison with those obtained by existing diagnostic measures and which, early in the disease, may be superior. By adopting a technique which virtually eliminates the demonstration of naturally occurring ox cell antibodies, a one-tube test can be used, thus permitting the testing of very large numbers of sera. No absorptions are required and the difficulties inherent in selecting an arbitrary titre as a standard of abnormality are substantially reduced. If the protean manifestations of infectious mononucleosis take second place only to those of syphilis, it follows that the disease should be excluded with a regularity approaching that of syphilis. The use of this simple test would facilitate such investigation, but it is recognized that its application on a far wider scale is necessary before its value can be fully assessed.

V. SUMMARY

It is suggested that routine tests for infectious mononucleosis in a large number of apparently unrelated syndromes would disclose a frequent common aetiology and perhaps modify the present conception of the course of the disease.

A technique is suggested for a simple test involving the demonstration of ox cell haemolysins which would make the testing of large numbers of sera practicable.

The results in sixty cases of infectious mononucleosis and 200 controls are

evaluated. The test does not reduce significantly the proportion of serologically negative cases but may confirm the diagnosis before the characteristic sheep cell agglutinins are demonstrable.

The test is as sensitive and as specific as other serological tests for infectious mononucleosis and is very much easier to perform.

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APPENDIX

Case histories

Case 2. A case of ambulatory infectious mononucleosis with slight sore throat and generalized glandular enlargement, presented signs and symptoms sufficiently clear to warrant an immediate diagnosis on clinical grounds. The blood film was not seen in our laboratory but was reported as 'typical of glandular fever'. A routine ox cell haemolysin test was positive and reported as confirming the diagnosis. Subsequent analysis of the serum showed a sheep cell agglutinin titre of 1:64 which was completely removed by both guinea-pig kidney and boiled ox cells. The ox cell haemolysin titre was 1:8. Six weeks later the sheep cell titre was 1:16 and the ox cell haemolysins negative at 1:2. Recovery was complete.

Case 19. This apparently unsatisfactory case was found, while in the neuropsychiatric ward, to be running a mild pyrexia. Routine haematological examination showed a leucocytosis of 12,000 cells per cu.mm. of which 69% were atypical mononuclear cells. A few glands were palpable in the axillae but the patient felt perfectly well. He was sent on sick leave for his nervous complaint and during this time sent three specimens of serum, only the last of which showed a positive ox cell test. One month later he had been released from the Service after a thorough medical overhaul. The possibility of acute leukaemia can therefore be excluded.

Case 23. There is no doubt that this was a definite clinical and haematological case of infectious mononucleosis. Inquiry elicited the fact that the first serum sent had been taken in convalescence. The sheep cell agglutinin titre was 1:32 which was unaffected by absorption with ox cells but reduced to 1:5 by absorption with guinea-pig kidney. The ox cell haemolysin test was negative. A possible explanation is that the ox cell titre had fallen to normal early in the disease. There was some evidence that the stimulation of sheep cell antibodies was specific in that 14 days later the titre had fallen to 1:8. The case is placed in the serologically positive group with hesitation.

Case 24. An airwoman aged 19 reported having a mild sore throat and dysphagia for 3 days. A swelling in the neck had been present but no glands were palpable when she was seen. A throat swab revealed no pathogenic organisms; the total and differential white cell counts were quite normal. Serum examination showed sheep cell agglutinins to a titre of 1:16 completely absorbable by ox cells and guinea-pig kidney; ox cell haemolysins were present at 1:32. One week later the titres were 1:16 for sheep cells and 1:8 for ox cells. This may have been a late

case of mild infectious mononucleosis, but, with no evidence to support the diagnosis, it must be included as a false positive result.

Case 25. Clinically, a case of infective hepatitis in an officer aged 45. The white cell count showed polymorphonuclear leucocytes 2200, lymphocytes 1650 and monocytes 250 per cu.mm. with no abnormal cells. Sheep cell agglutinins to a titre of 1:32 were present which were completely absorbable by ox cells and guinea-pig kidney; the ox cell haemolysin titre was 1:8. Ten days later the titre was 1:8 for sheep cells and no ox cell haemolysins were demonstrable. This is unquestionably a false positive result.

Case 26. A classical retro-pharyngeal abscess in an airwoman of 18. Sheep cell agglutinins of the same nature as in the foregoing cases were present to a titre of 1:32, ox cell haemolysins were found to a similar titre. One week later the sheep cell titre was the same, that for ox cells being 1:64. It is possible but unlikely that this was a case of pyogenic infection superimposed on infectious mononucleosis.

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