

"SOME SEROLOGICAL ASPECTS OF INFECTIOUS
MONONUCLEOSIS WITH SPECIAL REFERENCE TO
THE USE OF AGGLUTININ-ABSORPTION TESTS
IN THE DIAGNOSIS"

by

George Dempster, M.B. Ch.B., B.Sc.

*Lewis Cameron Postgraduate
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The serological diagnosis of infectious mononucleosis was introduced by Paul and Bunnell, (1932), who demonstrated the presence of an agglutinin and haemolysin for sheep's erythrocytes in the serum of cases; these were found in high concentration during the acute stage of the disease, the amount of haemagglutinin corresponding to that of haemolysin, a rather unusual finding, as haemolysins are usually developed to a much greater extent than haemagglutinins. The presence of low concentrations of apparently similar antibodies in normal human serum had long been recognised. At first no qualitative distinction was drawn between these antibodies but soon a controversy developed concerning their respective natures. A definite qualitative difference between the antibodies present in infectious mononucleosis and in normal human serum can be shown to exist by placing them in contact with various tissue extracts and erythrocytes of different species of animals, and observing whether the antibody is bound, or absorbed, by these substances; such absorption tests, as they are called, can be used to differentiate the respective antibodies. In explanation of the use of absorption tests in infectious mononucleosis reference must be made to the work of Forssman, (1911), and the subsequent extension of his findings by other workers.

Forssman discovered that he could produce a powerful haemolytic antibody for sheep's erythrocytes by the intraperitoneal injection of a/

a watery extract of the organs of the guinea-pig into a rabbit. Materials from other animals, the horse and cat, were also shown to be capable of producing this antibody, but extracts from the organs of the ox and rat failed to do so. Forssman, and others, divided the animal species into two classes, according as to whether emulsions of their organs had this antigenic power. Animals which fell into the same group as the guinea-pig were known as 'the guinea-pig type', those which did not as 'the rabbit type'. The haemolysin produced in response to the injection of organs from animals of 'the guinea-pig type' was quite distinct from the normal haemolysin which resulted from the injection of sheep's erythrocytes into the rabbit, for the latter could not be absorbed by guinea-pig kidney or by any other heterophile antigen. The term 'heterophile' was introduced by Friedemann (1917) and is used to describe Forssman's haemolysin and so distinguish it from the ordinary sheep cell haemolysin resulting from the injection of the homologous antigen; this latter haemolysin he called 'isophile'. It should be pointed out that there are other heterophile antibodies than Forssman's, but that the latter may be identified by its affinity for Forssman heterophile antigen. This antigen has been shown to be present in greatest amount in guinea-pig kidney and lung, and therefore the former is often used for absorption tests. Friedemann compared the properties of 'isophile' and/

'heterophile' haemolysin thus:-

	Sensitises the Erythrocyte of:	Is absorbed by:	Is not absorbed by:
Isophile haemolysin	Sheep, goat, <u>and</u> ox	sheeps erythrocytes (fully), goat, and ox erythrocytes (partially)	Organs containing heterophile antigen i.e. those of 'the guinea-pig type'
Heterophile haemolysin	Sheep, goat, <u>not</u> ox	sheeps erythrocytes, heated sheeps erythrocytes, organs contain- ing heterophile antigen.	Ox erythrocytes, Organs of 'the rabbit type'

In his own experiments he demonstrated that the haemolysin for sheep's erythrocytes present in normal human serum was absorbed by substances known to contain Forssman heterophile antigen, such as heated sheep's erythrocytes (the heating destroys the 'isophile' but not the 'heterophile' antigen), guinea-pig kidney emulsion, and horse kidney emulsion, but not by substances lacking this antigen such as emulsions of ox liver, pigs liver, and human kidney. He concluded, therefore, that the normal haemolysin for sheep's erythrocytes in human serum was heterophile in nature.

The early workers with rabbit heterophile antiserum failed to demonstrate its agglutinating power. This failure was found by Trou-Hia-Hsu, (1922), to be due to the fact that the heterophile agglutinating antibody will only react with sheep's erythrocytes which have been allowed to stand for 2-3 days. When such cells were used, agglutination, which was just as specific as the corresponding haemolytic reaction, occurred. In addition/

addition, he found that heating the fresh cells or treating them with alcohol, ether, or osmic acid, did not render them agglutinable, nor could the agglutination be attributed to bacterial contamination of the cell suspension. On this account directions for the technique of the Paul-Bunnell test usually stressed that the sheep's corpuscles should not be used in the fresh state; this of course implied that the antibody in infectious mononucleosis behaved in the same manner as the normal heterophile agglutinin.

The assumption that the haemolysin and haemagglutinin in infectious mononucleosis sera was of the Forssman heterophile character was commonly held until Bailey and Raffel, (1935), attempted to absorb the antibodies from 3 cases of the disease, using heterophile absorbing reagents. The antibodies, haemolysin and haemagglutinin, resisted this treatment but were absorbed by either fresh, or heated, erythrocytes of the ox and sheep. These results showed that the antibodies were neither 'heterophile' nor 'isophile' in character, (see table), and therefore quite distinct from any of the recognised sheep haemolysins, or haemagglutinins, including that present in normal human serum.

In the serological diagnosis of infectious mononucleosis by the direct Paul-Bunnell test, the serologist relied upon finding the agglutinin titre for sheep's erythrocytes raised above what was considered to be the normal level. Apart/

Apart from disagreement with regard to what constituted the normal level, the difficulties involved in interpreting this reaction were further manifested when it was appreciated that a certain number of cases received serum therapy, often because the anginal symptoms suggested the possibility of diphtheria or scarlet fever. Consequent upon serum therapy, as demonstrated by Davidsohn, (1930-1933), a heterophile antibody for sheep's erythrocytes is developed 10-12 days following the injection of horse, or even rabbit (Schiff 1937), serum. Davidsohn noted also that this antibody was present in higher concentrations in cases which developed serum sickness. Taniguchi (1922) suggested that the heterophile antigen present in the horse serum reacted with the normal heterophile antibody in the patient's blood, and that this produced the signs of serum sickness. On the other hand Powell & Jamieson, (1935), found the same proportions of cases of serum sickness amongst cases treated with horse serum previously absorbed with heterophile antibody, as with cases given unabsorbed serum. But whatever relationship this heterophile antibody had to serum disease, the fact remained that it might be present along with the infectious mononucleosis antibody, or mistaken for it.

It became imperative to distinguish between these different antibodies, and absorption tests were therefore devised with this aim. Stuart, Fulton, Ash, and Gregory, (1936), found that the/

the haemagglutinin produced by the administration of horse serum could be absorbed both by Forssman and non-Forssman antigens, e.g. they were absorbed both by guinea-pig kidney and by heated ox cells; in contrast the haemolysin was absorbed only by Forssman antigen. With guinea-pig kidney emulsion and heated ox erythrocytes, as absorbing reagents, Stuart, Welch, Cunningham, and Burgess (1936) were able to differentiate between normal sheep's haemagglutinin, the haemagglutinin found after serum administration, and that present in infectious mononucleosis as follows:

Antibody present in	Result of treatment with guinea-pig kidney suspension	Result of treatment with heated ox cell suspension
Normal Serum	Absorbed	Not absorbed
Serum Sickness	Absorbed	Absorbed
Infectious Mononucleosis	Not absorbed	Absorbed

Davidsohn, (1937), described results with a similar technique and Beer, (1936), investigated the absorptive powers of various other erythrocytes. Barrett, (1941), used the same reagents as Stuart, but found that the previous methods were not very suitable for tests upon normal sera where the initial titres were so low that it was difficult to decide whether or not absorption had taken place. With a modified technique he examined 300 normal sera/

sera, 31 sera from cases of infectious mononucleosis, and 7 sera from serum therapy cases. This modified technique of Barrett was employed in the work which is now presented.

This investigation into the serological diagnosis of infectious mononucleosis started after Halcrow (1943) had reported an outbreak of the disease at Larbert E.M.S. Hospital. Over the following two years a careful watch was kept, and whenever there was a possibility of this condition, serum was submitted for examination. In most cases the test was repeated with at least one further specimen. Of the 170 sera analysed in this report, 110 were from Larbert and the clinical diagnosis was made by Dr. Halcrow. The remaining 60 cases were patients in Bangour E.M.S. Hospital, and the Edinburgh City, Eastern General and Western General Hospitals. Dr. Halcrow and the writer were given full access to the case records and were given any information required concerning the illness; in this way the clinical analysis of the cases was made as uniform as possible. All the serological work recorded in this report was carried out by the writer. The following are the aims of the investigation:

1. To show that the correlation between the clinical diagnosis and the serological result using the absorption test is high, and that the latter is of the greatest value in establishing a diagnosis especially in late and/
and/

and relapsed cases.

2. To estimate the limitations of the direct Paul-Bunnell test.
3. To investigate the amount of normal heterophile (Forssman) agglutinating antibody in sera submitted for Routine Wassermann tests and compare it with that found in sera submitted for the Paul-Bunnell Test.
4. To investigate a series of serum therapy cases with Barrett's absorption technique.
5. To record the variations in titre during the course of the illness in as many cases as possible.
6. To obtain further information with regard to the conditions of the test and the behaviour of the antibody concerned in the reactions.

TECHNIQUE

The patient's serum was heated at 56°C for $\frac{1}{2}$ -hour to destroy complement so as to obviate haemolytic effects. The Direct Test (Paul-Bunnell). The following modification was used. Doubling dilutions of the serum ranging from 1/16 were made. To each of these dilutions an equal volume 0.5 c.c. of a 1% suspension of washed sheeps red blood cells was added. The usual control with saline, instead of serum, was included. The tubes were shaken and incubated for 4 hours in a 37°C incubator (initially 2 hours in a 37°C water-bath was employed). The test was placed in the ice-chest overnight/

overnight, and read the next morning. During the course of the investigation it was found that it was better to make a further reading after placing the test at 37°C for a period of 2 hours; in this paper only the cold temperature titres are recorded. The last tube showing macroscopic agglutination, after gently shaking the tube, and viewing the contents through a thinklayer produced by tilting the tube, was taken as the end-point.

The Absorption Test (Barrett's modification).

The Absorbing Reagents.

Guinea-pig kidney emulsion - About 50 pairs of guinea-pig kidneys were taken, decapsulated and separated from adjacent fatty and connective tissue. The parenchyma was then cut into small pieces and thoroughly washed in running water to free it from blood, and then finely sieved. The sieved tissue was autoclaved and suspended in 0.5% phenol-saline, the proportion of tissue in the suspension being 1 in 6 (by volume). The same suspension was used throughout the investigation during which time it was stored at 5°C.

Ox red cell suspension - The fresh ox cells were washed thrice in normal saline and resuspended in 3-4 times their packed volume of normal saline. This suspension was autoclaved, and then strained through muslin when cool. Sufficient saline was added to make up the proportion of cells in the suspension to 1 in 5; an equal volume of 1% phenol-saline was added and the reagent was then stored at 5°C. A 1 in 10 dilution of this stock suspension/

suspension was used in the test.

The Test.

This was carried out using 3 x $\frac{1}{2}$ inch tubes. To 1 c.c. of the patient's serum was added 0.3 c.c. of guinea-pig kidney suspension. The suspension was added in three successive doses of 0.1 c.c. and each dose was allowed to act for forty minutes, this made the absorption more effective. By the treatment the serum was diluted by the fluid portion of the guinea-pig suspension, and the supernatant which was obtained by centrifuging the mixture represented a 1 in $1\frac{1}{4}$ dilution of the original serum. 0.5 c.c. of the treated serum was added to 1.5 c.c. of normal saline and by this means a dilution of 1 in 5 was obtained. A preliminary test for the presence of sheep cell agglutinin in the treated serum was then carried out with 0.5 c.c. of this 1 in 5 dilution. The method employed to test for antibody was to centrifuge 0.5 c.c. of the serum dilution with 0.5 c.c. of an 0.2% suspension of sheeps erythrocytes at 2,000 r.p.m. for ten minutes. (With this technique no incubation was required and much time was saved). A reading was made by gently tapping the tube and observing whether the disc of cells which had formed could be resuspended; when agglutination had taken place the cells could not be uniformly resuspended. If the preliminary test with the 1 in 5 dilution showed that no agglutinating antibody was present then the agglutinin-absorption reaction for infectious mononucleosis was considered to be negative.

When/

When the preliminary test indicated that an agglutinating antibody remained in the serum treated with guinea-pig kidney, doubling dilutions of treated serum were made and the titre of the antibody estimated. The same method of testing for agglutination as was used for the preliminary test was employed throughout. The dilutions were prepared in such a manner that two series of dilutions could be set up at once (vide infra) and whilst the one (A) was used for testing the titre of the antibody, the other (B) was used for the second part of the absorption test, which consisted in showing whether the antibody could be absorbed by heated ox cells.

Two rows of tubes (A and B) were set out so that tube 1 of A was opposite tube 4 of B. The following diagram shows the dilutions as they were prepared, each tube containing 0.5 c.c. of the dilution (tube 1 of B excepted).

	lin $\frac{1}{4}$	lin $2\frac{1}{2}$	lin5	lin10	lin20	lin40	lin80	lin160 etc.
A	-	-	-	1	2	3	4	5
B	1	2	3	4	5	6	7	8

Into each tube of row A was put 1 c.c. of saline. Into tube 1 of row B was put 0.3 c.c. of treated serum (dilution 1 in $1\frac{1}{4}$). Into tube 2 of row B was put 0.25 c.c. of treated serum plus an equal amount of saline (dilution 1 in $2\frac{1}{2}$). Tube 3 of row B contained the 1 in 5 dilution prepared for the preliminary test and which now had 1.5 c.c. present in it. (0.5 c.c. having been removed for the preliminary/

preliminary test). The two sets of doubling dilution were then prepared by removing 1 c.c. from tube 3 of B into tube 1 of A., and mixing it with the 1 c.c. of saline already present in this tube, thus making a 1 in 10 dilution. Before transferring 1 c.c. of this 1 in 10 dilution to the next tube in row A, to prepare the 1 in 20 dilution, 0.5 c.c. was transferred to the adjacent tube in row B (tube 4). This was repeated with tube 2 of row A and with subsequent tubes until a sufficient series of dilutions had been prepared. The number of dilutions required naturally depended upon the titre of the antibody.

To each tube in row A was added an equal volume (0.5 c.c.) of an 0.2% suspension of sheep erythrocytes. These tubes were centrifuged and read as described above. The number of the last tube showing agglutination was noted, and the correspondingly numbered tube in row B was selected, together with the tubes on either side of it, to test for ox cell absorption. To the three selected tubes, 0.5 c.c. of ox cell suspension was added, the mixture shaken and immediately centrifuged. 0.5 c.c. volumes of supernatant fluid were pipetted off into fresh tubes and these were then tested for agglutinating antibody. If two out of the three tubes tested showed no agglutination after treatment with the ox cell suspension then the antibody was considered to have been absorbed. The method of selection of the tubes for ox cell absorption/

absorption makes allowance for the dilution made necessary by the addition of ox cell suspension, thus ensuring that failure to demonstrate agglutination following absorption was not due merely to dilution of the series beyond its end titre.

Interpretation of the Test.

Sera which gave (1) a titre of 1/40 or over following absorption with guinea-pig kidney, and (2) no agglutination in 2 out of the 3 tubes treated with the ox cell antigen were considered to be positive.

RESULTS.

1. The correlation of the ultimate clinical diagnosis with the result of the absorption test in a series of 170 patients.

Thirty-eight of these patients were ultimately diagnosed to be cases of infectious mononucleosis. The clinical diagnosis of infectious mononucleosis was based upon the occurrence of a characteristic blood picture accompanied either by fever, glandular enlargement, sore throat, or a combination of these symptoms. In addition to the main clinical feature, which was either fever, glandular enlargement or sore throat, other fairly constant symptoms were noted. These were lassitude and weakness, profuse sweating, headache and inability to concentrate. Secondary infection of the pharynx in the anginous type of case was in most cases due to Vincent's infection but infections with the haemolytic/

haemolytic streptococcus and pneumococcus also occurred. Many cases could be recognised as infectious mononucleosis from clinical and haematological investigation alone, provided that the blood picture was typical - W.B.C. 8-20,000, and a mononucleosis of 45-60% with many abnormal lymphocytes present. These cells of the 'glandular fever type' had a cytoplasm which was more basophilic than that of the normal lymphocyte, and, in addition, the cytoplasm was often vacuolated. Their nuclei varied greatly in shape and the chromatin was dense and often fenestrated.

For the purpose of estimating the degree of correlation between the ultimate clinical diagnosis and the result of the absorption test the cases have been grouped in the following manner:-

	<u>Ultimate Clinical Diagnosis</u>	<u>Absorption Test</u>	<u>Number of cases</u>
Group I	Typical case of I.M.*	+	17
Group II	Typical case of I.M.	-	10
	Typical case of I.M.	+	3
Group III	Atypical case of I.M.	+	8
Group IV	Not I.M.	-	132

Group I: These cases were clinically and haematologically typical, and, therefore, the agglutinin-absorption reaction, which was positive in each case, was merely confirmatory. Seventeen cases were of this nature. In two of them the titre with the direct test was inconclusive (below 1/256) and, therefore, the absorption test was/

* Infectious Mononucleosis.

was of value in establishing a definite positive serological result.

Group II: Cases which were clinically and haematologically infectious mononucleosis, but were serologically negative or inconclusive when examined by the absorption technique. There were thirteen cases in this group - 34% of the cases diagnosed as infectious mononucleosis. With three of these a trace of agglutinating antibody was observed after absorption with guinea-pig kidney, but the titre was less than 1/40 and, therefore, inconclusive. The probable explanation of this was that the serological examination was made late in the course of the illness; in Case 23 during the fifth week (see Table V), and in the other two cases after six and thirteen weeks respectively. It would be hardly justifiable to include these three cases in a sero-negative group; they were omitted, therefore, in the estimation of the percentage of sero-negative cases. As for the remaining ten cases, which were serologically negative, eight of them were examined during the first three weeks of the illness, that is, during the period in which a positive agglutinin absorption reaction might be most expected (see page 28). Admittedly in five of these cases only one examination was performed but in the other three the test was repeated. One case was consistently serologically negative when examined upon the second, twenty-first, and twenty-eighth days of illness. These ten cases, therefore, form the sero-negative group of cases of infectious mononucleosis/

mononucleosis in this series, and they represent 26% of the cases diagnosed as infectious mononucleosis.

Group III: Those cases in which either the blood picture was inconclusive or there were unusual clinical findings rendering the diagnosis difficult. In this group the absorption test was of considerable importance in establishing the diagnosis of infectious mononucleosis; short clinical notes will be given in these cases to demonstrate this. A fuller account has been given of one case which was particularly puzzling.

Case 8. Admitted with otitis externa of one months' duration, apart from this the only other abnormal clinical findings were enlarged glands in the neck and right axilla. The lymphocytosis was not high (only 32% of 7,000 W.B.C. per cu.mm.) but typical "glandular fever cells" were found. The agglutinin-absorption reaction was positive (titre 1/40), this finding was of great value in confirming the haematological diagnosis.

Case 24. Admitted with a month's history of cough, weakness, sweating, and loss of weight. Seven days before admission developed a stomatitis. Pyrexia developed three days after admission. The agglutinin-absorption reaction was shown to be positive three days later/^{titre}(1/80). Six days later the glands in the posterior triangles of the neck were found to be moderately enlarged. At the time of the serological test the blood picture was as follows: W.B.C. 8,000, Polymorphs 49%, Lymphocytes/

Lymphocytes 40%., Monocytes 8%., Abnormal Lymphocytes 3%. A positive agglutinin-absorption reaction was obtained before any glandular enlargement was observed.

Cases 26 and 28. These cases had on admission given a history of a recent attack of infectious mononucleosis. The agglutinin-absorption reaction was positive when tested on admission and was of value in confirming a diagnosis of relapsed infectious mononucleosis.

Case 10. Clinically this case appeared to be a severe case of acute haemolytic streptococcal tonsillitis (the haemolytic streptococcus was isolated) with cervical adenitis; no other glands were involved. The blood picture revealed an absolute lymphocytosis with atypical lymphocytes present. In this case the agglutinin-absorption reaction (positive titre 1/80) was of great value in establishing the diagnosis.

Case 20. The patient was admitted because of a sudden attack of lower abdominal pain accompanied by severe dysenteric symptoms. Bacteriological examinations of the stools were negative. He recovered from this and then developed enlargement of the cervical and right axillary glands. The spleen was also enlarged. The blood was typical of infectious mononucleosis. W.B.C. 8,200., Polymorphs 50%., Large Lymphocytes 15%., Small Lymphocytes 4%. Monocytes 9%., Abnormal Lymphocytes 22%. In this case/

case the unusual alimentary symptoms immediately prior to the glandular enlargement made the case atypical. The agglutinin-absorption reaction was positive titre 1/80.

Case 16. This was not a typical case and was clinically like infective hepatitis; there were, however, a rather larger number of abnormal lymphocytes than might be expected in this condition and the agglutinin-absorption reaction was positive titre 1/40, thus confirming a diagnosis of infectious mononucleosis with jaundice.

Case 15. An extremely puzzling case.

On admission:- Ten days history of a febrile illness with painful tender muscles, pain in the chest, sore throat, headache, anorexia, jaundice and epistaxis. Icteric index was 119 and the Van den Berg test gave a direct immediate positive result. Two months previously he had had an attack of gonorrhoea which was successfully treated.

Examination revealed fairly marked jaundice, enlarged tender glands, in the left axilla, liver slightly enlarged, and tenderness over the spleen. There was no evidence of cardio-vascular disease. Blood: W.B.C. 16,900., Polymorphs 73%, Lymphocytes 16%, Monocytes 11%. All the lymphocytes were of the abnormal type, as seen in infectious mononucleosis. The adenitis became generalised and therefore serum was submitted for a Paul-Bunnell Test. The agglutinin-absorption reaction was/

was carried out on the seventeenth day of illness and was found to be positive (titre 1/80); in view of the history and clinical findings the possibility of leptospiral jaundice was also considered and an agglutination test was performed at the same time - a titre of 1/640 was recorded.

Meanwhile aortic systolic and diastolic murmurs had developed and this raised the question of a bacterial endocarditis. Progress thereafter may be summarised as follows:-

(See over)

Day of illness	Clinical condition	Laboratory findings
18	Marked deterioration. Temp. swinging to 104°F. Rigors and profuse sweating. Adenitis gone. 50 c.c. antileptospiral serum given I.V.	Blood culture - negative Wassermann - negative G.C.F.T. - negative
20	Condition improved. Less jaundiced. Spleen markedly enlarged. 20 c.c. antileptospiral serum given I.V.	Trace of bile in urine
23	Aortic murmurs changing in character. Sulphadiazine given.	Icteric index 42. Van den Berg-direct negative. Blood culture - negative
29	Jaundice disappeared	W.B.C. 12,800 Polymorphs 82% Lymphocytes 7%
33	Condition deteriorating rapidly. Face puffy. Cardiac murmurs still present.	Urine - Many pus cells, R.B.Cs. and casts. Much albumin present.
36-7	Oedema of the ankles	W.B.C. 11,500 Polymorphs 81% Lymphocytes 16% Monocytes 2% Blood urea 200 mgm % Absorption test titre 1/160. - resisted absorption by ox cell antigen.
46	Semicoma, generalised oedema.	
52	Patient died.	

A post-mortem was carried out by Professor Drennan. ^sVegetation/were found upon the aortic cusps; these were shown to contain Gram-positive diplococci. Sections of the spleen and liver were stained by Levaditi's method but no leptospira were found. Sections of the kidney showed the lesions of an acute glomerulo-nephritis. Death was due to a subacute bacterial endocarditis and acute glomerulo-nephritis. In the clinician's view the/

illness was complicated by a leptospiral jaundice and an attack of infectious mononucleosis. The result of the agglutinin-absorption reaction was evidence in favour of the latter condition being present in the early stages of the illness.

Group IV: The cases in which a diagnosis of infectious mononucleosis was at one time considered owing to certain clinical or haematological findings. The agglutinin-absorption reaction was carried out in many cases of infective hepatitis and atypical pneumonia because, as is usual in these conditions, a certain proportion of abnormal lymphocytes were found in the blood; also included were cases of Vincent's angina, sore throat, tuberculosis, malaria and other febrile illnesses which were undiagnosed at the time of the serological investigation. In these cases the serological result was negative and a diagnosis other than infectious mononucleosis was ultimately made. It is conceivable that a certain proportion of this group might have been atypical cases of infectious mononucleosis which were serologically negative, but clinically as well as serologically the evidence was against such a diagnosis.

Included in this group was a case which gave an inconclusive result with the agglutinin-absorption reaction. The following is a brief note upon it:-

Case 30. This was not a case of infectious mononucleosis but one of chronic tuberculous adenitis/

adenitis, confirmed by biopsy, in a patient who had been treated for sixteen months for pernicious anaemia, by intramuscular injections of liver extract. An inconclusive agglutinin-absorption reaction was recorded (titre 1/10). The interest lies in the fact that Berstein (1940) has recorded a high Paul-Bunnell titre in a patient following parenteral administration of liver extract.

2. An estimation of the limitations of the direct Paul-Bunnell test based upon the results of agglutinin-absorption tests carried out upon all sera showing agglutination with the direct test.

Out of the hundred and seventy patient's sera ninety-one showed an agglutination titre of 1/32 or over by the direct Paul-Bunnell test.

Absorption tests upon these ninety-one sera showed that those giving a titre of 1/256 or over also gave a positive agglutinin-absorption reaction for infectious mononucleosis, and were clinically typical cases of that disease. Case 23 (see Group II) was an exception to this for the titre after absorption was reduced to 1/20 (an inconclusive result). Of those giving a titre of 1/128 by the direct test ~~six~~ gave negative absorption tests for infectious mononucleosis.

It was evident from the above findings that a titre of less than 1/256 could not be considered as diagnostic of infectious mononucleosis in this series of cases. Table I shows the distribution of the titres recorded in these ninety-one patients/

patients, and the proportion of these which gave a positive agglutinin-absorption reaction for infectious mononucleosis.

Table I

Titre (Direct Test)	Number of Sera	Number giving positive agglutinin-absorption reaction.
Diagnostic 1/256 or over	15	14
(1/128)	13	4
Inconclusive (1/64)	33	5
(1/32)	30	1
Total with titre less than 1/256	76	10

From Table I it will be seen that seventy-six patients' sera gave a titre of less than 1/256 (serologically inconclusive) but only ten of these gave a positive agglutinin-absorption reaction for infectious mononucleosis.

In comparison Table II shows how the application of the agglutinin-absorption test reduced the number of inconclusive serological results.

Table II

Titre after absorption with guinea-pig kidney	Number of sera
Positive i.e. 1/40 or over	25
Inconclusive { 1/20	4
{ 1/10	6

In only ten cases was an inconclusive result recorded using the agglutinin-absorption test, and three of these were clinically typical cases of infectious mononucleosis; two others had received serum therapy and a third (Case 30) had received

liver injections as already mentioned. In all the cases in which the agglutinin-absorption reaction was recorded as positive, the antibody was absorbed by the ox cell antigen. In two of the serum therapy cases (Case 15 and J.F.) the antibody resisted absorption with the ox cell antigen, and this also happened with one other patient who had no history of serum therapy.

3. A comparison of the amount of normal Forssman heterophile antibody found in the sera submitted for the Paul-Bunnell test with that found in a series of sera submitted for the routine Wassermann test.

For this purpose the thirty-five sera which contained antibody not absorbed by guinea-pig kidney (see Table II) were excluded. The remaining one hundred and thirty-five are compared with a series of one hundred and twenty sera, submitted for routine Wassermann tests. This comparison was made because it was noted that the Forssman heterophile antibody titres, which were being recorded for cases in which the diagnosis was ultimately shown to be other than infectious mononucleosis, were higher than usual;* it was necessary, therefore, to demonstrate that this was not due to the technique being used. The results are recorded in Table III.

Table III/

*Smeall, 1942.

Table III

Titre of Forssman antibody.	Sera submitted for:-			
	Paul-Bunnell test.		Wassermann Test	
	Number	%	Number	%
1/128	6	4.4.	0	-
1/64	25	18.5	5	4.2
1/32	26	18.6	15	12.5
less than 1/32	79	58.5	100	83.3

4. The results of agglutinin-absorption reactions in cases receiving serum therapy.

Serum was submitted from three cases with a history of recent serum therapy. The results obtained with these sera are recorded in Table IVa. In case 15 (the history of which has been given in some detail) infectious mononucleosis had been suspected clinically and confirmed serologically before the administration of the serum; three weeks after the injection the heterophile antibody resisted absorption by both guinea-pig kidney and ox cells - prior to the serum therapy the antibody resisted absorption only by guinea-pig kidney. The serum from one of the other two cases (M.T. and J.F.) similarly resisted absorption by both antigens; clinically these two patients were considered to be cases of serum disease.

Table IVa/

Table 1Va

Results of Absorption Tests upon
Serum Therapy Cases

Ref. No.	Antiserum Dose in c.cs.	Route	No. of days since serum was given	Reciprocal of Titre Direct Test	of Titre After treat- ment with guinea-pig kidney	Result of ox cell absorption
Case 15	70	I.V.I.	21	256	160	Not absorbed
M.T.	10	I.V.I.	10	64	10	I.S.*
J.F.	5	I.M.I.	10	128	20	Not absorbed

Because of these unexpected results sera were obtained from patients convalescing from diphtheria who had received antitoxic serum. The results obtained with these sera are recorded in Table 1Vb.

Table 1Vb/

*Insufficient serum to carry out
this absorption

Table IVb

Ref. No.	Antiserum		No. of days since serum was given	Reciprocal of Titre		Result of ox cell absorption
	Dose in c.cs.	Route		Direct Test	After treatment with guinea-pig kidney	
S 2	5	I.M.I.	18	64*	I.S.∅	I.S.
S 16	5	I.M.I.	26	32*	0	
S 12	8	I.M.I.	28	64	0	
S 15	8	I.M.I.	34	512	320	unabsorbed
S 1	5	I.V.I.	24	0		
S 3	5	I.V.I.	13	256	20	unabsorbed
S 7	5	I.V.I.	23	256	80	unabsorbed
S 6	5	I.V.I.	26	128*	20	I.S.
S 21	5	I.V.I.	49	128	0	
S 9	6	I.V.I.	21	512 H.T.	640	unabsorbed
S 13	6	I.V.I.	34	64	20	unabsorbed
S 10	6	I.V.I.	36	128	20	I.S.
S 11	20	I.V.I.	18	256	320	unabsorbed
S 8	20	I.V.I.	21	128	40	unabsorbed
S 22	20	I.V.I.	32	64	10	I.S.
S 14	20	I.V.I.	36	64*	0	

None of the cases showed signs of serum sickness

* Agglutination disappeared upon warming at 37°C for 2-4 hours.

∅ I.S. Insufficient Serum to carry out this absorption.

H.T. Highest dilution prepared from that serum.

From Table IVb it can be seen that titres

of/

of 1/256 and over were reached in serum therapy cases with the direct test, but that in the majority of these cases the titre was considerably reduced by treatment with guinea-pig antigen and absorption by the ox cell antigen was not demonstrated. This behaviour distinguished the antibody present from that occurring in the cases of infectious mononucleosis, for in the latter condition there was no reduction in titre by treatment with guinea-pig kidney antigen, and the antibody was readily absorbed by the ox cell antigen. In most of the serum therapy cases a titre of at least 1/32 was demonstrable and in some (S2, 16, 12, 14) it appeared to be due to normal heterophile antibody. It would appear that a titre of 1/40 or over after guinea-pig kidney absorption may be expected in cases receiving intravenous or intramuscular horse serum.

5. Variations in the titre during the course of the illness in cases of Infectious Mononucleosis.

The variations in titre of infectious mononucleosis antibody can be followed only by the absorption test since reactions in low dilutions recorded by the direct test may be due to normal heterophile antibody. This has been done and the results presented in Table V. In some cases only a single specimen has been taken. It can be seen at a glance that the highest titres are reached about the second or third week and that as a rule there is a steady fall so that quite a/

a proportion may be expected to show titres of 1/40 or less after ten or eleven weeks. The second point to note is that definite high-titre and low-titre cases can be recognised. In one low-titre case (C 5) the titre was zero at the sixth week, and in another (C 8) at the third week.

Table V/

-30-
TABLE V

CASE NUMBER	RECIPROCAL OF THE TITRE AFTER ABSORPT ⁿ WITH GUINEA-PIG KIDNEY DURING THE 1 st , 2 nd , 3 rd , ... etc. WEEK OF ILLNESS												
	1	2	3	4	5	6	7	8	9	10	11	12	16
C3	2560	1280	640	-	320	-	-	-	-	-	20	0	-
CI	2560	-	2560	2560	-	-	-	-	-	-	-	-	160
C27	-	-	-	-	-	-	-	-	-	-	-	-	1280
CII	-	1280	1280	-	1280	-	-	320	160	160	80	-	-
C6	-	5120	-	-	-	-	-	640	-	-	-	-	-
CI2	-	1280	640	320	320	-	-	-	-	-	-	-	-
CI9	1280	-	640	-	-	-	-	-	-	40	-	-	-
C2	320	-	320	-	160	-	-	-	20	-	-	-	-
C25	-	-	-	-	-	-	-	-	320	-	-	-	-
CI3	-	640	320	160	-	-	-	-	-	-	-	-	-
CI7	160	-	640	-	-	-	-	-	-	-	-	-	-
C4	160	80	-	-	-	-	-	-	-	-	-	-	-
CI4	-	160	160	-	-	-	-	-	-	-	-	-	-
CI5	-	-	80	GINEN ANTI-SERUM	* 160	-	-	-	-	-	-	-	-
C5	-	-	80	-	40	0	-	-	-	-	-	-	-
C8	10	40	0	-	-	-	-	-	-	-	-	-	-
CI6	-	-	40	-	-	-	-	-	-	-	-	-	-
C20	-	-	-	40	-	-	-	-	-	-	-	-	-
C23	-	-	-	-	20	10	0	-	-	-	-	-	-
C24	-	-	-	-	80	-	-	-	-	-	80	-	-
C26	-	-	-	-	-	-	-	-	-	80	-	-	-

* NOT ABSORBED BY OX CELL ANTIGEN

6. Observations upon the properties of sera containing

"Infectious Mononucleosis Antibody"

The direct test was used to make the following observations.

(a) Degree of thermostability of the Antibody. The serum from Case 1 was heated at 55°C for six hours, and no change in the titre (1/512) was observed; after twenty-seven hours, however, little activity remained. Heating at 37°C for similar periods produced no alteration in titre. The serum showed very slight loss of activity after storage at 5°C for over a year.

(b) Conditions affecting the sheeps red blood corpuscles.

1. Age. With the serum from Case 25 no difference in the titre (1/256) was noted when fresh cells were used instead of cells kept for one day.

11. Cells from different breeds of sheep. Cells from Suffolk, Cheviot and Oxford breeds, as well as from lambs, were tested fresh, and one day after collection, with serum from Case 25. No significant difference in titre was found.

(c) Effect of temperature. Parallel tests were carried out, one wholly at 5°C, the other wholly at 37°C. This was done with serum from case 1 and also with serum from case 3. The tests were read at intervals of $\frac{1}{2}$ -hour from the first two hours, and hourly thereafter.

At 37°C. The highest titre was reached after two hours.

At/

At 5°C. The highest titre was reached after six hours and this titre was lower than that of the parallel test at 37°C.

(d) Tests for agglutination of an organism containing Forssman Antigen.

The organism used was a strain of B.dys. Shiga known to contain Forssman antigen. Agglutination tests were carried out using the sera from cases 1, 4, 14, and 26. No reaction was observed in any of dilutions (1/30 - 1/480) prepared. Sera containing normal heterophile antibody (titre 1/128) also gave no reaction.

DISCUSSION

in 66% of the cases of infectious mononucleosis the agglutinin-absorption reaction was positive, in 26% of the cases this reaction was negative, and in the remaining 8% of the cases an inconclusive result was recorded. These facts were obtained by correlating the ultimate clinical diagnosis with the serological result in each case. In about two-thirds of the cases in which the reaction was positive the diagnosis could have been made from the clinical and haematological findings alone, in the remaining one-third of the cases the results of the agglutinin-absorption reactions were of undoubted value in establishing the diagnosis.

One of the main purposes of this investigation was to estimate the limitations of the direct Paul-Bunnell test, and the results obtained by agglutinin-absorption tests have been used to do this. Few will deny that a high titre Paul-Bunnell reaction/

reaction (direct test) is significant in the diagnosis of infectious mononucleosis, but with low titres there is difficulty in finding agreement upon the level at which the titre becomes significant. If only a high-titre were accepted, e.g. 1/256, a certain number of cases would be excluded - in this series ten out of twenty-five cases; if, on the other hand, a low titre were accepted, e.g. 1/64, a large number of cases not suffering from infectious mononucleosis would be included - in this series thirty out of the one hundred and seventy patients. Even with this low titre cases might be missed and yet be revealed by the agglutinin-absorption test - one of the cases reported fell into this category - moreover, cases of infectious mononucleosis in which the normal heterophile antibody has been increased to, or beyond, this low titre would then be falsely classified as serologically positive; this would have occurred in eight cases of this series if a titre of 1/64 had been accepted as diagnostic. It should be noted that of the twenty-five cases of infectious mononucleosis found positive by the absorption test, only fourteen gave a significant titre in the direct Paul-Bunnell test.

We have had to differentiate between the heterophile antibody present in infectious mononucleosis, in normal sera, and that which may be present in patients receiving serum therapy. Considering first the normal heterophile antibody
it/

it was noticed that a higher average titre was recorded for the sera submitted for the Paul-Bunnell test than for sera requiring routine Wassermann tests, and this was excluding the cases which gave positive agglutinin-absorption reactions and were finally considered to be infectious mononucleosis. The explanation may be two-fold: in the first place the sera submitted for the Paul-Bunnell test were mostly from patients with acute febrile illnesses, whilst those for the Wassermann test were from all kinds of cases including normal pregnant women. Secondly there was the age factor, the majority of the cases of suspected infectious mononucleosis were in the 20-30 age group. The routine Wassermann cases covered a wider range. G.H. Smith (1937-8) found that out of four hundred and one sera from people between the ages of 1-30 years, only four were completely lacking in heterophile antibody, but as the age-group rose the normal titre fell. This might explain why such a high percentage of cases showed titres of 1/32 or 1/64 and it is possible that the normal heterophile antibody is non-specifically increased in all acute febrile illnesses, infectious mononucleosis included. In infectious mononucleosis, of course, we realise that another heterophile antibody may be present. (There was one case recorded here in which the normal heterophile agglutination reached a titre of 1/256 and yet the titre of the "infectious mononucleosis antibody", as demonstrated by the agglutinin/

agglutinin-absorption test, was only 1/20). This means that before a diagnostic level for the Paul-Bunnell test could be fixed, it would be necessary to find the highest level reached by the normal heterophile antibody in acute febrile illnesses which were clinically not glandular fever. Although not recorded, fifteen cases of syphilis in the acute stage were tested (eight with adenitis, seven with jaundice), and of these eight gave a titre of 1/32 and two (both jaundice cases) gave a titre of 1/128, this was considered to be further evidence of the non-specific stimulation of the normal heterophile antibody in acute febrile illnesses.

The titres recorded for the direct test throughout are low temperature titres (i.e. read immediately after removal from the refrigerator). It was noted, as reported by Stuart et al (1934), (1935) that low temperature titres for infectious mononucleosis and "serum sickness" sera were only one tube higher than titres at 37°C. Whereas normal serum titres might show a titre of 1/64 or 1/128 with strong agglutination at refrigerator temperature which disappeared at 37°C in $\frac{1}{2}$ -1 hour - a drop of at least 2-3 tubes. On this account it is probably better not to refrigerate the direct test but to make final readings after four hours' incubation at 37°C.

Only a few sera were received from patients who had had serum therapy. With the present technique/

technique complete absorption by the antigens was not demonstrated. In case 15, for example, a positive agglutinin-absorption reaction was demonstrated before the administration of serum but subsequent to the injections an antibody was present which resisted absorption by both antigens. (Barrett, himself, only tested seven specimens from patients who had received serum therapy and the titres in these cases before absorption were not much above the normal range; moreover he stated that "positive absorption tests in patients who may be suffering from serum sickness should at present be regarded as of doubtful significance").

Many of the sera obtained from convalescent diphtheria patients who had received antitoxin gave similar results. It was noted that absorption with guinea-pig kidney/^{antigen}reduced the titre considerably; there was the possibility that this reduction was only apparent, and was due to the absorption of normal Forssman antibody which had been increased in titre by the injection, and was present in greater quantity than the serum sickness antibody. If this had been the case one would have expected to find a marked fall in the titre of the direct test when it was subsequently warmed to 37°C., and although this was observed in some of the cases, e.g. S.6, it was not in others, e.g. S.3 or S.7. It must be concluded therefore that the 'serum sickness antibody' was/

was partially absorbed by guinea-pig kidney antigen. A similar reaction was reported in one case by Barrett (1941). The writer's experience with Barrett's test would indicate that the heterophile antibody present in serum sickness was incompletely absorbed by both antigens; moreover it was recognised in this test by the fact that it was only partially absorbed by both antigens. The agglutinin absorption tests described and used by other workers apparently demonstrated full absorption with both antigens.

Resistance to absorption with guinea-pig kidney antigen was noted in 2 cases with no history of infectious mononucleosis or of serum therapy. In one case (Case 30) the patient was receiving liver injections which are said to give rise to heterophile antibody (Bernstein 1940). In the other case the antibody resisted absorption with both antigens - Barrett reported this in a few cases and because of it insisted upon the use of both antigens in the test.

The manner in which the titre altered with the duration of the illness can be seen from Table V; altogether the titres from twenty-one cases were recorded and the general picture indicated that high titres were reached early in the course of the illness, and that the titre might still be rising during the second and third weeks - the highest titres were reached/

reached between the end of the second or third weeks. Following this the titre gradually subsided, and it was possible to find a residual titre in some cases three or four months after the onset of the illness. Case 27 was exceptional, for a high titre was found after fifteen weeks of illness. In some cases a very high titre was recorded (1/2560), in others the titre remained at a much lower level.

"Infectious mononucleosis antibody" behaved like a true antibody as judged by its variation in titre during the course of the illness, its degree of thermostability, and its ability to act at low temperatures. It would appear that the antibody will react with fresh erythrocytes to the same titre as with erythrocytes which have been kept for one day. (The keeping of the erythrocytes for one day is a procedure often recommended in directions for the Paul-Bunnell test but this is only likely to increase the agglutination effects produced by normal Forssman antibody). As Mutermilch (1924) had reported finding an individual sheep whose erythrocytes lacked the Forssman antigen, the erythrocytes from different breeds of sheep were tested with 'infectious mononucleosis sera' with the object of finding out whether they differed in their agglutinability by 'infectious mononucleosis antibody'. Although no differences were found, a much more extensive investigation/

investigation of this question would have to be made before coming to a decision. The 'infectious mononucleosis antibody' caused no agglutination of an organism known to contain the Forssman antigen - as might be expected - but it was thought that normal sera containing antibody of the "Forssman type" might show some action; however none was observed.

It is hoped that this small series of cases will emphasize the need for applying absorption methods in the routine diagnosis of sera submitted for the Paul-Bunnell test. At least an abridged agglutinin-absorption test with only one antigen should be employed, and the double absorption technique could be reserved for difficult cases such as those known to have received serum therapy.

SUMMARY.

1. The correlation is high between the clinical and the serological diagnosis of infectious mononucleosis when the absorption test is used. (66%).
2. The direct Paul-Bunnell test is of limited value especially in late and relapsed cases.
3. In high-titre cases, e.g. 1/256, the direct test will suffice, but a considerable number of cases may never reach such high titre.
4. Definite sero-negative cases occur; they form a low percentage of this series. (26%).
5. Variations in titre during the illness have been recorded.

6. An application of Barrett's absorption technique to a series of cases which had received serum therapy did not give satisfactory results, for complete absorption of the antibody, by either of the antigens employed was not demonstrated.
7. There appears to be a non-specific stimulation of the normal heterophile antibody in acute febrile illnesses.
8. It is suggested that an abridged absorption test should be employed in the routine serological diagnosis of infectious mononucleosis.



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