Immunology, 1961, **4**, 142.

A Two-Stage Indirect L.E. Cell Test

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(*Received 8th July*, 1960)

Summary. The technique and results of a two-stage indirect L.E. cell test are described.

An account of the course of the lupus phenomenon as it occurs in this test is given.

Details are presented of a patient with systemic L.E. whose direct reaction was persistently negative in the presence of a strongly positive indirect reaction.

INTRODUCTION

The Lupus Erythematosus (L.E.) cell was first described in bone-marrow preparations from patients with systemic L.E. by Hargraves, Richmond and Morton in 1948. Haserick, Lewis and Bortz (1950) showed that lupus cells could be produced by incubating normal bone marrow with γ globulin from an L.E. patient; and Miescher and Fauconnet (1954) suggested that this γ globulin represented an antibody to a component of the cell nucleus. This antibody is now generally known as the 'lupus factor' and the reaction whereby lupus cells are formed in its presence as the 'lupus phenomenon'.

It has been clear for some time that the lupus phenomenon occurs in two stages (Rifkind and Godman, 1957; Nathan and Snapper, 1958; Aisenberg, 1959). In the first stage the lupus factor reacts with nuclei from dead white cells. In the second stage lupus cells are formed from these altered nuclei by living polymorphs.

A two-stage L.E. cell test has been devised on this basis. This test has proved more sensitive than others that have been tried. It has also been found useful for studying the course of the lupus phenomenon and for inhibition experiments.

In this paper the method is described and results obtained by its use with human sera are presented.

MATERIALS AND METHODS

SERA. Sera for testing were stored at $-20°$, in some cases for 2 years. Neither prolonged storage nor repeated freezing and thawing appeared to cause much loss in the activity of the lupus factor.

SUBSTRATE. For diagnostic use a 'white-cell rich plasma' is used. 20 ml. of blood, freshly taken from a normal donor, are defibrinated. 2 ml. of 6 per cent dextran* are added to each 10 ml. of defibrinated blood. The mixture is allowed to settle in a siliconed test tube held at 45° from the vertical for half an hour at 37°. This sediments the great majority of the red cells and the white-cell rich plasma is pipetted off. A white-cell count is made and should be between 4000 and 6000/c.mm. The white-cell rich plasma is then placed in small tubes, frozen in a dry ice and acetone mixture, and stored at $-20°$. Each tube is

* 'Dextraven' (Benger).

thawed only once, immediately before use. 0.1 ml. of this preparation is used in each test and provides a supply of dead and broken white cells, to act as substrate, as well as a source of complement.

PHAGOCYTIC SYSTEM. If only a few tests are to be performed, the living white-cell preparation is made by allowing the cells to crawl out of two drops of blood, freshly taken by finger prick and left to clot on a clean glass slide in a moist chamber at 30° for 1 hour. (A half-hour incubation at 37° can also be used but there is some tendency for the edges of the clot to dry.) After incubation the clot is lifted off with forceps and the remaining red cells washed away with some drops of fresh human or guinea-pig serum (not with saline, which tends to damage white cells). This leaves a monolayer of polymorphs and monocytes on the slide, which is then used at once.

An Improved Method for preparing White-Cell Monolayers

For a larger number of tests, or where more than one batch of tests is to be done on one day, the following method is more convenient. 10 ml. of blood are collected by venepuncture into a test tube containing 0.25 ml. 8 per cent disodium versenate, 0.25 ml. 8 per cent trisodium versenate, and 1 ml. 6 per cent dextran. This mixture is allowed to settle in a 45° rack for half an hour at 37° and the supernatant fluid — a white-cell and platelet rich plasma — pipetted off. At this stage the preparation can be kept at 4° all day and even overnight without damage to the white cells. When the phagocytic preparations are to be set up, an aliquot is warmed to room temperature and mixed with one-tenth of its volume of a solution containing 2 g. $CaCl_2$ and 2 g. $MgCl_2.6H_2O$ in 100 ml. Two drops of this mixture are put on a clean slide and allowed to clot for 1 hour at 30° in a moist chamber. After removal of the plasma clot excess fluid is drained off and replaced by a drop of fresh human or guinea-pig serum.

This technique gives very satisfactory monolayers and has been used for all the more recent tests.

THE TWO-STAGE INDIRECT L.E. CELL TEST. *First stage.* 1. 0.1 ml. (or more) of the test serum and 0.1 ml. of substrate are incubated in small glass tubes at 37° for 1 hour.

2. At the same time the phagocytic preparation is being incubated at 30°.

3. The serum-treated substrate preparation is centrifuged at 300 *g* for 2 minutes. The supernate is discarded and the pellet resuspended in 2 drops fresh normal human or guinea-pig serum. If the test serum proves to be very anti-complementary or toxic for living white cells, or if for any reason it is wished to separate the two stages completely, the pellet is washed once in fresh normal human or guinea-pig serum before resuspending.

Washing in saline or repeated centrifuging damages white cells (living or dead) and gives rise to atypical reactions.

Second stage 4. The drops of serum-treated substrate are put on to a freshly prepared phagocytic film; a coverslip is placed carefully on top, with care to avoid air bubbles, and is sealed to the slide with a rim of wax to prevent drying.

5. The preparations are now incubated in moist chambers at 37° for a further hour. During this time the reaction may be observed under the phase-contrast microscope, but this has not been found a reliable method of reading the tests.

6. After 1 hour the cover slips are removed, surplus fluid drained off and the films dried in air. They are then stained by the Leishman method and examined.

A positive control with a known lupus serum is included in each batch of tests.

RESULTS

APPEARANCE OF THE REACTION

At the End of the Sensitization Stage

At the end of the sensitization stage smears were made of the serum-treated substrate (after procedure 3 of the method) with many positive and negative sera.

When the final test is negative most of the 'nuclei' at this stage are small (5–10 μ diameter) and stain densely blue; some, however, are somewhat swollen (10–15 μ diameter) and stain a reddish purple. A chromatin pattern is occasionally to be made out and cytoplasmic tags are not infrequent. A limiting membrane can usually be distinguished.

In a positive test (including very strong and typical examples) the great majority of the nuclei are in no way different from those described above. Occasionally a small pro-

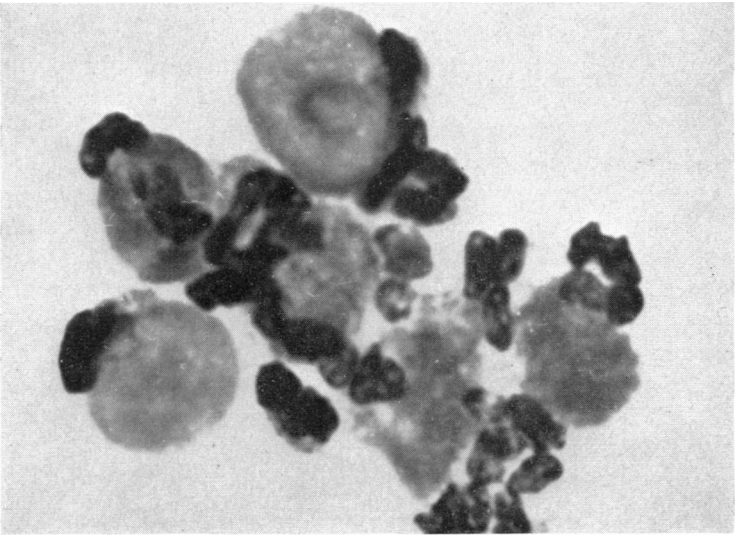

FIG. 1. A group of L.E. cells and a rosette showing the mottled appearance of the inclusions (× 1800).

portion, however, are recognizably 'globs' (Carrera, Reid and Kurnick, 1954) as judged by the following criteria, all of which must be fulfilled.

(i) The nucleus must be swollen — at least 10 μ across.

(ii) There must be no chromatin pattern. Though often so described neither 'globs' nor lupus cell inclusions are truly homogeneous. When seen under high magnification the appearance is mottled — as if the nucleohistone has been so much diluted by the inflow of protein that it no longer covers the whole area (Fig. 1).

(iii) There must be no cytoplasmic tags.

(iv) There must be no external limiting membrane.

The number of 'globs' found at the end of the first stage bears no direct relation to the final strength of the reaction.

At the End of the Test

Negative slides show an evenly distributed monolayer of polymorphs, monocytes and eosinophils with a few red cells scattered among them.

Clumping of the white cells occurs around particles of foreign matter and if any degree of drying occurs. If neither of these causes is operative strong white-cell clumping can be regarded as evidence of the presence of a white-cell antibody in the test serum. Clumping is in no way specific for L.E., and has been used, for example, as a criterion for titrating a rabbit anti-leucocyte serum (Wildy and Ridley, 1958).

Damage to the white cells may be shown as vacuolation, 'smudging' or detachment. If any of these changes is widespread the test should be repeated; the substrate pellet should be washed free from sensitizing serum at procedure 3. This is hardly ever necessary.

Positive slides show the three characteristic products of the lupus reaction: the haematoxyphil body (or 'glob'); the lupus rosette and the lupus cell.

The usual morphological criteria are used for the rosette and the lupus cell (*see* Marmont, 1959). The criteria for a 'glob' have been listed above.

The relative proportion of rosettes (often with 'globs') on the one hand and lupus cells on the other varies markedly.

A number of factors seem to play a part in this variation including the relative proportions of lupus factor and its substrate, and of sensitized substrate and phagocytic cells, and the abundance of complement. Some sera always give predominantly rosette reactions. In particular this was found with some strongly anti-complementary sera (for example M. S. and R. L.) and in sera from patients with the clinical diagnosis 'Rheumatoid Arthritis with L.E. cells' (e.g. B. H. and A. F.). In these cases the central (haematoxyphil) part of the rosette was often larger than normal.

QUANTITATIVE ASSESSMENT

There is too much variation in different parts of the field to make counts of lupus phenomena per 1000 polymorphs meaningful and it has been found useful to score reactions arbitrarily in this way:

Negative	−	No lupus phenomena present.
Weak	(+)	Lupus phenomena present, but in best region less than one per high power field.
Positive	+	Best region has at least one lupus phenomenon per high power field.
Strong	++	In best region 10–50 per cent of polymorphs are involved in lupus phenomena.
Very strong	+++	In best region over 50 per cent of polymorphs are involved in lupus phenomena (Fig. 2).
	(+++)	Slightly weaker than +++.

However, if a quantitative result is wanted, this is obtained by titrating the serum (in fresh guinea-pig serum) and taking the highest dilution giving a ++ result. If the neat serum is weaker than ++ the titration can be performed backwards by using larger amounts of serum in the first stage.

In this series no serum has been found to give positive results with 1 ml. aliquots if the reaction with 0.2 ml. was negative, and 0.2 ml. has usually been used for diagnostic

purposes. However, if there is ample serum available, it is probably as well to use a 1 ml. aliquot. In this way it is hoped to find very weak reactors who may give negative direct tests.* Strong sera have titres of up to about 1/40.

THE ROLE OF COMPLEMENT IN THE LUPUS REACTION

During the first stage of the reaction complement is fixed if it is present, but the reaction is not inhibited by its absence. The latter may be shown either by using a heated test serum and washed white cells suspended in heated serum before freezing, or — more satisfactorily — by performing the first stage in the presence of versene. In both cases satisfactory reactions were obtained.

The requirement of complement in the second stage is similar to that of other phagocytic

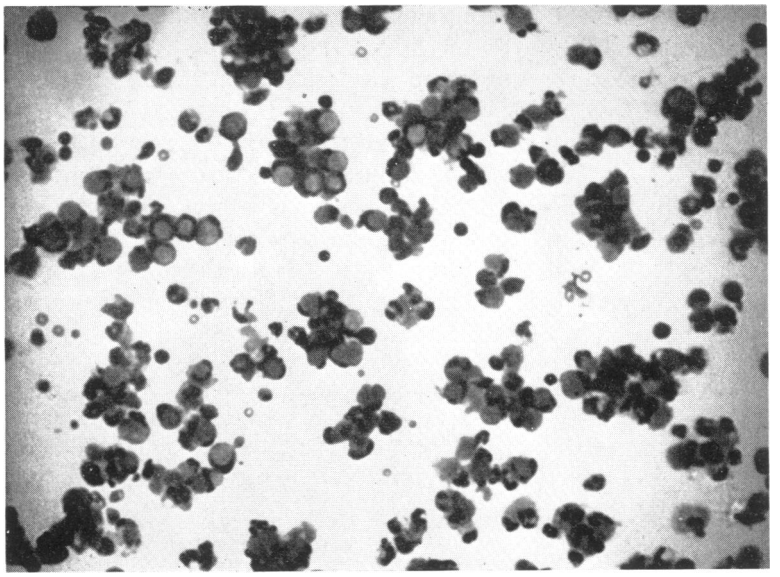

FIG. 2. A +++ Indirect L.E. Phenomenon (×200).

reactions. If a strong sensitizing serum was used no requirement of complement could be shown and the reaction was equally strong no matter whether the monolayer was suspended in treated or fresh serum or even in 1.5 per cent γ globulin which is rather anti-complementary.

For weak sensitizing sera the absence of complement in the second stage — and, particularly, the use of the γ globulin — led to weaker reactions and an increase in the number of 'globs' and rosettes as compared to L.E. cells. These reactions then resembled those found with anti-complementary sera.

Versene cannot be used in the second stage as it takes the white cells off the slide.

Bovine conglutinin had no effect on the L.E. reaction.

This difficulty in showing a requirement for complement and other phagocytosis-

* More recent experience with a larger group of patients on the service of Dr. H. G. Kunkel at the Rockefeller Institute has shown that such weak reactors are not too infrequent.

promoting serum factors (Aisenberg, 1959) is found in other phagocytic systems using monolayers, probably because similar serum factors are involved in the process of attachment of the white cells to glass.

RESULTS OF DIAGNOSTIC TESTS WITH HUMAN SERA

Tables 1 and 2 give details of results obtained.

Negative results have been obtained with a number of human sera sent for diagnostic purposes and several normal sera.

Positive results have been obtained with twenty-six samples of serum, one pleural fluid and one percardial fluid from seventeen patients. Of these fourteen were suffering from

TABLE 1

DETAILS OF PATIENTS GIVING A NEGATIVE INDIRECT L.E. CELL TEST

In all these cases the direct test was negative also.

Description	*No. of cases*	*Indirect L.E. cell test*
Normal sera (laboratory staff)	10	All −ve
Rheumatoid arthritis	15	All −ve
Felty's syndrome	1	−ve
Sarcoid	1	−ve
Serum sickness	1	−ve
Leucopenia — unknown cause	1	−ve
Hyper gammaglobulinaemia — unknown cause	1	−ve
Discoid L.E.	6	All −ve
Myelosclerosis	1	−ve
L.E. in remission	3	All −ve
Serum containing sperm autoantibody	1	−ve

clinical systemic L.E., two had severe rheumatoid arthritis without evidence of systemic L.E. and one had a complex illness with features of Waldenström's macroglobulinaemia as well as of systemic L.E. Most of this material was received as serum so that the results of the direct L.E. test had usually to be taken from the notes. Nevertheless the information seems to show that the results of the direct and indirect tests run parallel in sixteen of the seventeen patients.

A CASE OF SYSTEMIC L.E. WITH NEGATIVE DIRECT TEST

The seventeenth patient (C. A.), a twenty-year-old unmarried girl, presented in June 1959 with a clinical syndrome compounded of a bowel illness which clinically, sigmoidoscopically and on rectal biopsy was indistinguishable from a relatively mild, ulcerative colitis; a mild and transient rheumatoid type of arthritis; and bruising due to thrombocytopenia.

Investigations on patient C. A. during admission to hospital, June 1959.
Hb. 13.0 and 14.4 g. per cent. W.B.C. 8.900 and 10,000/mm.^{3} Normal differential count.
Platelets 60.000 and 100,000/mm.^{3}
E.S.R. (Wintrobe) 23 and 45 mm./hr.

TABLE 2

DETAILS OF PATIENTS GIVING A POSITIVE INDIRECT L.E. CELL TEST

Patient	*Sex*	*Age*	*Diagnosis*	*Samples*	*Direct test*	*Two-stage test (0.1 ml. aliquot)*
K. C.	F	26	Florid SLE	6 samples from August 1957 to December 1959	Always +ve	+++
F. P.	M	46	Florid SLE in 1957	1 serum and pleural fluid 1957	+ve	+++
			Complete remission	Serum 1959	−ve	−
I. H.	F	53	Florid SLE 1957	Serum 1957	+ve	+++
			Remission 1959	Serum 1959	(+)ve	+
O. W.	F	43	Florid SLE 1957	1 serum and pericardial fluid	+ve	++
A. R.	F	49	SLE and hepatitis 1957	1 serum	+ve	++
			Remission 1958	Serum	−ve	−
R. L.	M	6	SLE 1958	Serum	+ve	+++
A. H.	F	35	SLE 1959	1 serum	+ve	+++
M. S.	F	40	SLE 1959	2 sera	+ve	+++ (atypical)
A. F.	F	63	Rheumatoid arthritis with L.E. cells	1 serum	+ve (atypical)	+ (atypical)
M. F.	F	44	SLE and macroglobulinaemia	1 serum	+ve	+++
C. A.	F	20	SLE	2 sera	−ve	++
A. B.	F	20	SLE	2 sera	+ve	++
G. H.	F	30	SLE	1 serum	+ve	+
G. L.	F	58	SLE	1 serum	+ve	+
A. S.	F	44	SLE	1 serum	+ve	+
E. P.	F	56	SLE	1 serum	+ve	++
B. H.	M	72	Rheumatoid arthritis with L.E. cells	2 sera	(+)ve	++ (atypical)

Serum Proteins and Electrophoresis Pattern Normal.
Bone marrow. Appearance consistent with Idiopathic Thrombocytopenic purpura.
Direct L.E. cell test. Negative on three occasions. The preparations showed marked white-cell clumping but nothing suggestive of L.E.
The Indirect L.E. cell test. Strongly positive (Titre 1/16).

Treatment was entirely symptomatic and no steroids were given.

Symptomatically she improved steadily and in January 1960 was symptom free except for slight bowel frequency.

The platelet count was 70,000 in October 1959 and 200,000 in January 1960; the E.S.R. 20 and 18 mm. (respectively). On both occasions the direct L.E. cell test was negative; the indirect test strongly positive.

There is little doubt then that this girl is suffering from systemic L.E. and has lupus factor in quite high concentration in her serum.

In attempts to discover why her direct test should be negative the following experiments were carried out.

[The prefix C. A. before a preparation denotes that it was prepared from the blood of the patient Miss C. A.]

TEST OF THE ANTIGENICITY OF C. A. WHITE-CELL NUCLEI

Washed polymorphs and monocytes were prepared from the patient's blood by the method of Wildy and Ridley (1958). These were suspended in fresh normal human serum and frozen and thawed.

This preparation was found to be a satisfactory substrate in the indirect test for C. A. serum and for other lupus sera. It therefore appears that her polymorphs are antigenically normal for this reaction.

TEST OF THE PHAGOCYTIC ACTIVITY OF C. A. WHITE CELLS IN THE LUPUS REACTION

Phagocytic monolayers were made from C. A. venous blood in the usual way, washed and resuspended in fresh guinea-pig serum. These were capable of forming L.E. cells from sensitized substrate from C. A. and from other lupus patients. Phagocytosis of *E. coli* was also normal.

TEST OF THE ANTI-COMPLEMENTARY ACTIVITY OF C. A. SERUM

C. A. serum had a normal complement titre and was not anti-complementary in either a haemolytic system using guinea-pig complement or a conglutinating system using horse complement. This is in contrast to the somewhat similar case of Formijne and Van Soeren (1959).

TEST OF AN 'ALL C. A.' SYSTEM

Both substrate and phagocytic preparations were made from the patient's blood in the usual way.

The substrate was incubated without adding any further serum, centrifuged and resuspended in a drop of its own supernate. The phagocytic preparation was resuspended in a drop of C. A. serum.

Surprisingly this 'all C. A.' test was strongly positive, and the paradoxical situation that this finding presents has not so far been fully resolved.

It was found, however, that the reaction became negative if the phagocytic preparation was left in its own clot supernate instead of being drained and resuspended in C. A. serum. This clot supernate was also inhibitory when used to resuspend another drained white-cell preparation.

This inhibitory factor was not present,

(*a*) in the 'clot supernate' of a normal donor,

(*b*) in the 'clot supernate' of C. A. serum mixed with normal white-cell and platelet rich plasma before recalcification,

(*c*) in the supernatant of C. A. white-cell and platelet rich plasma allowed to clot and retract completely in a tube.

The inhibitory factor therefore appears to be produced only under rather special conditions and to require the presence of some particulate component of C. A. blood — presumably white cells, since the direct test performed on defibrinated buffy coat (which contains no platelets) was also negative.

Its action did not appear to be a non-specific inhibition of phagocytosis since phagocytosis of red cells by an 'inhibited' preparation persisted. It appeared to represent either an inhibition of the change from sensitized nucleus to 'glob' or some other alteration in the substrate.

DISCUSSION

The lupus phenomenon is still the standard laboratory method for the diagnosis of systemic L.E. and there is no definite evidence that the particular antibody on whose presence it depends can be distinguished from the many other anti-nuclear antibodies that may be found in this disease by any other method.

For diagnostic purposes the lupus phenomenon is generally elicited by a 'direct test' using the patient's blood for the complete test. These tests, although easy to do, are often time-consuming to read, as in weak reactions only a very occasional L.E. cell is found. Indirect tests, which use the patient's serum only, have been introduced in considerable numbers in recent years (*see* Marmont, 1959), partly for experimental purposes and partly to find a more sensitive and easier diagnostic test. Schultz, Baum and Ziff used white-cell monolayers in 1953. Snapper and Nathan introduced the use of a separate substrate in 1955, and since this work was started, German and Huber (1958) have published a method using frozen white cells for this purpose.

Some form of two-stage method, such as the one described, is helpful for any attempt to analyse what is apparently a fairly complex reaction. It may be, however, that such a method has diagnostic merits as well, the requirements of the two stages being so different.

The first stage requires antigen (dead white-cell nuclei) and antibody (lupus factor) in proportions which may make it necessary to use a relatively large volume of a weak test serum.

The second stage requires a source of living and active leucocytes, the sensitized substrate and fresh normal serum (to supply complement and possible other factors), all in a small final volume. It does not require free lupus factor; and the presence of the not infrequently cytotoxic and anti-complementary lupus serum is not an advantage.

Furthermore it seems that in many cases the interaction of white-cell nuclei, lupus factor and complement produces damage to other living white cells (Lachmann, 1961). This effect is presumably of value in the production of the direct L.E. cell test, in which it is probably a way of obtaining substrate, but it is undesirable where maximum phagocytosis is desired.

For maximum phagocytic activity leucocytes should be on a surface rather than in suspension; should not be taken out of serum and should not be centrifuged. The attachment of leucocytes to glass is an active process and at the time of preparation all cells on the glass are alive.

For easy reading, it is preferable to be able to remove unused substrate from the slide, which is not possible if a dried white-cell smear is used as substrate.

Finally, for inhibition studies it is important to use a two-stage procedure. This allows inhibition due to competition for the antibody (the type of inhibition generally studied) to be distinguished from inhibition due to cytotoxicity and anti-complementary or phagocytosis-inhibiting factors.

From the experiments described the lupus reaction seems to describe the following course.

(i) REACTION OF LUPUS FACTOR WITH ANTIGEN

This reaction is not usually accompanied by any specific visible change in the substrate nuclei (a finding in agreement with the work of Nathan and Snapper, 1958).

(ii) CONVERSION OF SENSITIZED NUCLEI TO 'GLOBS'

Morphologically this change involves not only swelling of the nucleus with homogenization of its contents but the disappearance of the limiting membrane. The latter change seems to be responsible for the observation that 'globs' stick to glass and are found in the final preparations whereas unaltered nuclei are drained off. As previously reported by Nathan and Snapper (1958) but in contrast to the findings of Rifkind and Godman (1957) 'glob' production was found to occur to any extent only in the presence of intact white cells although occasionally a few 'globs' were found at the end of the first stage. It seems not unlikely that enzymes from polymorphs are involved in this stage.

(iii) PHAGOCYTOSIS

It is not clear if 'glob' formation is a necessary preliminary to phagocytosis or an alternative fate for the sensitized nuclei. The observations that lupus cell inclusion are often smaller than 'globs'; that, unless there are multiple inclusions, they are mostly round whereas 'globs' are usually somewhat ellipsoid or irregular; and that reactions showing many 'globs' may have few typical L.E. cells are in favour of the latter view.

It was hoped that observation by phase contrast would help to settle these questions; but this technique was found to be of little help in the author's hands. In the living polymorphs, the cytoplasmic granules are so much more phase dense than the nuclear material that it was often difficult even to make out a nucleus, let alone to make any detailed observations on its structure.

In the seventeen cases of this study one has been found to have a positive indirect test in the presence of a negative direct reaction. This did not reflect a greater sensitivity of the indirect test, as the serum contained an adequate quantity of lupus factor, nor was it due to the serum being anti-complementary, but apparently resulted from the production of an inhibitor of the second stage during the incubation of the patient's blood. How common this may be it is impossible to predict on the basis of one case, especially as much of this material was selected on the criterion of a positive direct test. It would seem to be well worth while to perform an indirect test on patients whose clinical picture suggests a diagnosis of systemic L.E. but whose direct reaction is negative.

ACKNOWLEDGMENTS

I should like to thank Dr. R. R. A. Coombs, in whose laboratory this work was done, for his invaluable help, interest and advice.

I am grateful to the Physicians of Addenbrooke's Hospital and Professor S. J. Hartfall of Leeds for allowing me access to their patients; and in particular to Dr. A. P. Dick and Dr. D'A Kok for permission to publish details of Miss C. A.

This work was done while holding the John Lucas Walker Studentship of the University of Cambridge and a Science Scholarship of the B.M.A. I wish to thank both these authorities for their support.

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