

after any one's name, Frimodt-Möller deserves the credit as he was the first to publish a report on the condition. As it has been observed independently by several medical men, impersonal nomenclature will be most appropriate and adequate.

### Summary

1. Symptomatology in 85 cases of pulmonary eosinophilosis is reviewed.
2. The acute and the chronic types are described.
3. The constant finding of high-titre cold agglutination is reported.
4. A short review of the available literature on the condition, and a discussion on the aetiology, are given.
5. That it is due to an infection, probably a virus, is also suggested.

My thanks are due to D.M.S. (India) for allowing me to publish the paper, to Colonel M. A. Jaferey for permitting me to study the cases and to Captain B. Natarajan for helping me in the investigation of cold agglutination.

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### COMPLEMENT-FIXATION TEST WITH WITEBSKY, KLINGENSTEIN, KUHN (WKK) OR SIMILAR ANTIGENS : AMODIFIED TECHNIQUE

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A TECHNIQUE of a complement-fixation test for kala-azar with WKK antigen was described by Greval, Sen Gupta and Napier in 1939. The value of this test in the diagnosis of kala-azar has been assessed in the subsequent publications by the writer (Sen Gupta, 1943, 1944). In the course of his investigations on the complement-fixation test for kala-azar using antigens prepared from the so-called leprosy bacilli of Kedrowsky and of Lleras, according to the WKK method, during the last two years, the writer has felt that certain alterations in

the technique were advisable in order to obtain the best results with these antigens.

In the technique described by Greval, Sen Gupta and Napier, the maximum amount of antigen not interfering with 1 MHD of complement was determined by titration and this was used in the test proper.

One volume of each of the gradually increasing dilutions, 1 : 10, 1 : 20, 1 : 30, etc., was titrated for anticomplementary activity by mixing with 1 MHD of complement in a volume and one volume of normal saline, and after proper incubation the presence of free complement was detected by adding one volume of sensitized cells and incubating for half an hour.

In the course of investigations it was found that the anticomplementary titre of the antigen was markedly different if instead of one volume of saline one volume of 1 : 25 dilution of a known inactivated non-anticomplementary negative serum was used during the titration of the antigen. The titre was very much lower when the titration was carried out in the presence of 1/25 dilution of the serum in the amount that is present during the test proper. Also the titre was higher in the presence of volume of serum in 1/100 dilution, and highest in the absence of any serum. The amount of antigen and complement free to react was different in the two dilutions of serum used in the test proper, and some slight irregularity was seen in some of the results obtained.

It was therefore decided to titrate the antigen for anticomplementary titre in the presence of 1/25 dilution of a 'negative' serum, to use only 1/25 dilution of the serum in the test proper (this ensures 'specificity' for kala-azar), and to modify the method of titration of the complement. To bring out the differences in the degrees of complement fixation it was decided to use the standard method of using two strengths of complement as in the Wassermann test. The sensitized sheep-cell suspension was prepared in accordance with the requirements of Wassermann test, method no. 4 of Medical Research Committee (1918) with the exception that the sheep-cell suspension was standardized according to a method described by Greval *et al.* (1930).

The technique that has thus been evolved has been tried in a large series of cases and has been found to be quite satisfactory. A brief description of the technique is given below :—

1. *Titration of the antigen.*—0.1 c.c. of the benzolic solution of the antigen is taken up with a 1 c.c. pipette and spread on a glass mortar and allowed to dry completely. 0.2 c.c. of normal saline is then added to the residue in the mortar and a suspension is made by trituration. This forms the starting point for making the dilutions 1/10, 1/20 upwards.

In a series of test tubes labelled to show the dilutions, 1/20, 1/30, 1/40, etc., up to 1/90, put 0.25 c.c. of the corresponding antigen dilution, 0.25 c.c. of 1/25 dilution of an inactivated negative serum, and 0.25 c.c. of complement dilution containing 1 MHD. Mix by gently shaking the tubes. Keep at room temperature for half an hour, then at 37°C. for half an hour. Add 0.25 c.c. of sensitized cells to each tube, mix,

incubate at 37°C. for half an hour; read for haemolysis. The strongest dilution of the antigen permitting a complete haemolysis corresponds to the anticomplementary activity short of 1 MHD of complement, and this is the dilution of the antigen to be used in the test proper.

This dilution is then tested for haemolytic activity by incubating 0.75 c.c. of this dilution with 0.25 c.c. of sensitized cell suspension. The antigen is usually found to be non-haemolytic.

The power of fixation of the antigen is tested by putting up comparative tests with a series of known sera using a known antigen and the new antigen.

Supposing that 1/60 is the working dilution arrived at by titration, in order to make up the dilution needed for the test proper, a total of 12 c.c. of saline will be required for the residue left after evaporation of 0.1 c.c. of the benzolic solution.

The titration of the antigen is not repeated daily, but the method of titration of the complement and the putting up of an antigen control during the test proper, serves to check the correctness of the antigen titre.

**2. Titration of the complement.**—1 : 10 dilution of the complement in normal saline is first prepared. From this 1/20, 1/30, 1/40, 1/50, . . . 1/120 dilutions are made. Two rows of tubes are set up on a rack to correspond to these dilutions of the complement. Two tubes, one behind the other, correspond to each dilution. In the tubes of the first row are placed 0.25 c.c. of the corresponding complement dilution and 0.5 c.c. of normal saline. In the tubes of the back row are put 0.25 c.c. each of the corresponding complement dilution, 1/25 dilution of inactivated negative serum, and the working dilution of the antigen. The tubes are kept at room temperature for half an hour, then incubated at 37°C. for half an hour. Then 0.25 c.c. of sensitized sheep cells is added to each tube and these are incubated at 37°C. for half an hour, after which the minimum haemolytic dose (MHD) of the complement is read. This is taken as the highest dilution at which there is complete lysis of the sheep cells. The tubes in the two rows usually show a similar degree of lysis. If there is a disagreement in the titre as indicated in the two rows, this is usually due to the defect in the antigen dilution, and can be rectified by again titrating the antigen against 1 MHD of complement in the presence of 1/25 dilution of a negative serum, and using the antigen in the new working dilution thus obtained.

**3. The test proper.**—The serum to be tested is inactivated to destroy the complement by heating to 55°C. for half an hour in a water bath. 1 : 25 dilution is prepared by mixing 0.1 c.c. of serum with 2.4 c.c. of normal saline.

The antigen dilution is made up as described previously (*vide supra*).

Two strengths of complement dilution are made so as to contain 2 MHD and 5 MHD in a volume. Suppose 1 : 80 is the titre (MHD) of the complement, a 1 : 40 solution of the complement will contain 2 MHD and a 1 : 16 solution 5 MHD in a volume.

Three tubes, placed one behind the other, are required for testing each serum; the first tube is for serum control with 2 MHD of complement, the second and the third for the test with the antigen and 2 and 5 MHD of complement respectively.

#### An ensemble

- Tube 1. Serum 1/25 dilution 0.25 c.c. + normal saline 0.25 c.c. + 2 MHD complement in 0.25 c.c.
- Tube 2. Serum 1/25 dilution 0.25 c.c. + antigen dilution 0.25 c.c. + 2 MHD complement in 0.25 c.c.
- Tube 3. Serum 1/25 dilution 0.25 c.c. + antigen dilution 0.25 c.c. + 5 MHD complement in 0.25 c.c.

Mix, leave at room temperature for half an hour, then in an incubator at 37°C. for half an hour.

Add sensitized sheep cells 0.25 c.c. to each tube, mix; incubate at 37°C. for half an hour. Read for haemolysis.

The following general controls are put up along with the test proper:—

1. Antigen control: 0.25 c.c. of each of the following: antigen dilution, 1/25 dilution of negative serum, 1 MHD of complement.

2. Negative serum control: same as 1 but 2 MHD of complement is used.

3. Positive serum control: a known positive serum put up as in the test proper.

4. Cell suspension control: 0.25 c.c. of cell suspension put up with 0.75 c.c. of normal saline.

#### The method of recording the results of the test

The degree in inhibition of haemolysis in the different tubes is read as follows:—

Complete inhibition of lysis	=	+	positive.
A trace of lysis	=	T	
More than a trace of lysis	=	±	{ doubtful.
Almost complete lysis	=	? -	
Complete lysis	=	-	{ negative.

A trace of lysis is detected by leaving the tube in a refrigerator overnight to allow the cells to settle down to the bottom of the tube.

The control tubes 1 and 2 should show complete lysis; the positive serum control should show no lysis with 2 and 5 MHD complement; the cell suspension tube should show no lysis.

Any serum that shows any inhibition of lysis in the serum control tube is to be regarded as anticomplementary; the other tubes are of no value; no opinion as to fixation of the complement by this serum in the presence of antigen can be given.

With complete lysis in the serum control tube, complete inhibition of lysis with 2 MHD of complement along with complete or almost complete inhibition (?T) with 5 MHD is reported as 'strongly positive'; complete inhibition of lysis with 2 MHD, with partial or no inhibition with 5 MHD, is reported as 'positive'; a trace of lysis or partial lysis with 2 MHD, with partial or no inhibition with 5 MHD, is reported as 'doubtful'; and complete or almost complete lysis with 2 MHD complement as 'negative'.

#### Significance

As pointed out in the previous publications on the subject of complement-fixation test for kala-azar by the writer, in the absence of severe (lepromatous) leprosy and post-kala-azar dermal leishmaniasis, a positive or strongly positive reaction with this test is indicative of kala-azar. It should be borne in mind, however, that a very small proportion of clinically obvious cases of chronic pulmonary tuberculosis gives a positive reaction. This is not a serious drawback. The doubtful reaction is regarded as an indication for further clinical and parasitological investigation. A negative reaction almost rules out kala-azar except in some very

early cases. A negative reaction is obtained in only about 1 per cent of all untreated cases of kala-azar seen in Calcutta.

#### *The advantages of this technique*

This technique has the advantage that the antigen and the complement are titrated under the exact conditions that exist in the test proper. The titrations are carried out in such a way that there are exactly 2 and 5 MHD of complement free to react. The adoption of the serum dilution of 1 : 25 ensures 'specificity' of the test for kala-azar as in the previous technique described by Greval, the writer and Napier. The use of two strengths of the complement in differentiating the degrees of positive reaction is in line with the standard British methods of the Wassermann test. Also, this technique is much less time-consuming than the previously described method, because only one dilution of the serum has to be made. The amount of complement required is somewhat more.

The writer is thankful to Dr. Dharmendra, officer-in-charge of the Leprosy Research Department, and to Mr. R. Bose, the chemist of the same department, for kindly preparing for him several batches of the antigens from the different acid-fast bacilli.

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### A CULTURE MEDIUM FROM GROUND-NUT MEAL

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ACTON *et al.* (1932) used a vegetable medium from a green variety of mung dal (*Phaseolus mungo*) for cultivation of the intestinal bacteria. Brewer (1943) suggested a mixture of soya bean and cotton seed or peanut meal for the preparation of a general purpose media using sodium sulphide as an activator; the sodium sulphide would readily generate sulphuretted hydrogen which by lowering the pH of the medium facilitates enzymatic proteolysis.

[Recently, Gottschall (1944) has noticed that during the digestion of meat, papain is activated by a sulphhydryl group from the meat substrate and therefore no preliminary activation is required.]

Groundnut is a good source of certain minerals and vitamin B complex (Pickett, 1942) particularly nicotinic acid; the meal obtained after extraction of oil contains a high percentage of protein body rich in certain essential amino-acids such as arginine, histidine and lysine (Basu, 1943) and it has been shown by Basu and Sen Gupta (1944) to undergo peptonization under the influence of various proteolytic enzymes. It was therefore considered that this meal might be used for preparing a medium for the cultivation of various organisms. The peptonization, however, is variable, but it can be easily increased by an activator. We have found sodium thiosulphate to serve this purpose quite well. The growth-promoting power of the medium might be further potentiated by the incorporation of a liver digest which is a good source of accessory growth factors.

#### Experimental

**Groundnut meal.**—The hydraulic-pressed cake of groundnut as available in the market was powdered in a pebble mill, passed through a 60-mesh sieve, dried and stored in a glass-stoppered bottle, special attention being paid to cleanliness in the whole process. The powder is fragrant and palatable, and consists of protein 49.6 per cent, ether extract 6.2 per cent, carbohydrate 22.08 per cent, fibre 5.6 per cent and ash 5.8 per cent.

**Preparation of the basal medium.**—150 gm. of the above meal powder containing 8.36 per cent nitrogen was taken in 1,500 c.cm. of water, and 200 c.cm. of Cole and Onslow trypsin extract was added and thoroughly shaken. The mixture was then incubated at 50 to 55°C. for four hours. It was filtered through a fine muslin cloth and the volume was made up to 1,500 c.cm. and the pH adjusted to 7.8. Any precipitate was filtered off and the clear filtrate was analysed for nitrogen content. The average result of analysis from three experiments was 0.4 per cent, or 47.8 of the nitrogen content of the meal.

The oxidizable matter of the above broth was then determined by the usual permanganate method in terms of milligrams of oxygen required for 100 c.cm., and was found to be 1.3 per cent (average). It was diluted with water to contain 0.75 per cent oxidizable matter. The final volume of broth obtained was 2.6 litres. The sodium chloride content of the broth was adjusted to 1.4 (cf. Scribner and Krueger, 1937).

**Preparation of papain digest medium.**—6.0 gm. of papain (Ceylon variety) and 3.0 gm. of sodium thiosulphate were mixed together and taken in about 100 c.cm. of water. This suspension was added to a mixture containing 150.0 gm. of meal powder in 1 litre of water. The volume was made up to 1,500 c.cm., and the pH adjusted to 6. The whole mixture was then incubated at 50 to 55°C. for 4 hours. The digested