Measurement of total haemolytic complement activity in body fluids

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SUMMARY The measurement of total haemolytic complement is of diagnostic value in the rheumatic diseases. A method which is easier to perform than that of Kabat and Mayer (*Experimental Immunology*, 2nd ed., Thomas, Springfield, Ill.) is described. A unit based on the mean value of the complement activities of normal sera is proposed to replace CH_{50} units. Other steps by which standardisation of the assay may be improved are suggested.

The lysis of a red cell by the complement system proceeds by a series of reactions of the complement components with different sites on the cell surface. Depletion of complement components of the serum or other body fluids in diseases may be detected by measuring the overall haemolytic activity, following a method such as that of Kabat and Mayer (1961). It is usual to express the results of such assays in terms of the 'total haemolytic complement (CH_{50}) units'.

The technique requires the measurement by pipette of quite large volumes of solutions, which makes the routine assay of moderate numbers of specimens tedious. Furthermore, some published observations are not in accord with the theory for the assay described by Kabat and Mayer (1961).

In the course of developing a simpler routine technique we were able to assess the validity of their assay.

Material

Alsever's solution was prepared as described by Kabat and Mayer (1961).

Complement buffer was prepared from Oxoid Complement Fixation Test Diluent Tablets (Oxoid Ltd, London).

Sheep blood was obtained fresh and without sterile precautions from the local abattoir. Blood (2 volumes) in Alsever's solution (1 volume) was stored at 4°C for not less than five days and no more than 15 days before use.

Rabbit Haemolytic Serum was purchased from Wellcome Reagents Ltd, Beckenham, Kent.

Sterile sheep blood from known donors was obtained from Tissue Culture Services Ltd, Slough, Bucks.

Apparatus

A Unicam SP 500 monochromator spectrophotometer, fitted with Gilford electronics and maintained to give accurate wavelength readings, was used for optical density measurements at 541 nm. Mechanical pipettes (Micrometerpette and Finnpiettes) were used to dispense liquid volumes up to $500\ \mu\text{l}$. A Lumix 6-MF dispenser (Chemlab Instruments Ltd, 1b Seven Kings Road, Ilford, Essex) was used for larger volumes. Polypropylene centrifuge tubes ($16 \times 110\ \text{mm}$ 10-12 ml, Medical Aids Dept, ICI Ltd, Pharmaceuticals Division, Alderley Park, Cheshire) were used as test vessels.

Method

Sheep cells were washed several times by suspension in 0.85% saline and centrifugation. A red cell suspension in complement buffer was prepared from packed washed cells according to Kabat and Mayer (1961). The final red cell concentration was adjusted so that $500\ \mu\text{l}$ of the suspension gave an OD_{541} of 0.70 ± 0.02 when lysed with 7 ml of distilled water. The sheep cells were sensitised by adding an equal volume of a predetermined concentration of rabbit haemolytic serum in complement buffer. Serum and joint fluid specimens were mixed with equal volumes of

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cold complement buffer. Aliquots were dispensed into 12-ml centrifuge tubes, followed by buffer and sensitised cells, according to the following protocol:

Tube No.:	1	2	3	4	5	6
Either 50/50						
serum/buffer (μ l)	10	15	25	35	50	75
Or 50/50 joint						
fluid/buffer (μ l)	25	35	50	75	100	125
Complement buffer			3.25 ml			
Sensitised sheep cell suspension				500 μ l		
				Vortex mix		

Incubate in closed water bath, 37°C 1 hour. Centrifuge (RCF value 1700 g 5 min). Measure OD of supernatant liquids at 541 nm against distilled water.

Duplicate blank and total lysis tubes, containing respectively 3.25 ml buffer and 3.25 ml water to which 500 μ l of sensitised sheep cell suspension had been added, were similarly treated.

Calculation of results

Percentage haemolysis was calculated from

$$\% \text{ Lysis} = \frac{\text{OD test} - \text{OD blank}}{\text{OD total lysis}} \times 100\%$$

A plot of % lysis against sample volume (μ l) on logit/linear paper (No. 7505 Chartwell, W. Heffer and Sons Ltd, Sidney Street, Cambridge), with % lysis as ordinate (logit scale), gave a smooth curve from which the volume of sample required to give 50% lysis was read directly.

Complement activity was expressed as a percentage of the mean value of a group of normal human sera determined in the same or similar experiment.

Results and discussion

In the technique of Kabat and Mayer (1961) the CH₅₀ unit of complement activity is defined as the volume of serum or fluid, in millilitres, which will lyse 50% of 5×10^8 optimally sensitised red cells under standard conditions of temperature, pH, etc. The cell suspension is not, however, standardised by counting, but by measurement of a completely lysed preparation at 541 nm, at which wavelength haemoglobin exhibits a very sharp peak. Spectrophotometric measurements of such a compound are particularly influenced by instrumental differences in bandwidth. Slight inaccuracies in wavelength calibration may also easily give errors of 5% or more in optical density measurements.

The definition of the CH₅₀ unit of complement activity given above takes no account of the known

variations between batches of sheep cells in their susceptibility to complement lysis (Walton and Ellis, 1958; Garratty, 1970). In confirmation of this earlier work, our results (Table 1) indicate that a change in red cell donor alone can give rise to nearly a two-fold difference in the values of CH₅₀. The Kent and Fife (1963) unit of complement activity is defined in a similar way. CH₅₀ units so defined make the clinical interpretation of laboratory results unnecessarily difficult.

Table 1 Variation of serum volume required for 50% lysis with different sheep red cells

	Sheep number					
	1	2	3	4	5	6
Volume of 50/50 sample required to give 50% lysis (μ l)	17.5	33	28.5	22.5	26	20

In order to arrive at a unit more satisfactory for routine purposes, we determined the complement activities of a group of seven normal (staff) sera, and calculated the mean and the activity of one of the sera as a percentage of this mean.

Portions of this serum, stored at -170°C (Garratty, 1970), served as a stable, internal reference standard, which was incorporated in every subsequent assay. Thereafter, all the results of our assays were reported as a percentage of mean normal serum activity. A freeze-dried preparation of human serum might be sufficiently stable to serve likewise as an international reference preparation, to facilitate inter-laboratory standardisation and the clinical interpretation of results.

This procedure reduced, but did not eliminate, assay variability. Difference, between sheep cells still gave rise to different complement activity values for the same specimen. Gewurz and Suyehira (1976) suggested that the incorporation of two standard sera can eliminate the influence of differences in red cell reactivity upon the results of the complement assay. It is evident from our results (Table 2) of experiments using randomly selected donor sheep and from those in Table 3 (modified from Bell *et al.* (1972)) that the suggested technique is ineffective; the relative haemolytic activities of different body fluid specimens can be strongly influenced by the particular source of sheep cells used. A consequence of this unpredictable behaviour of complement assays when unselected reagents are used is that the tests lose discrimination; the range of assay values found for normal specimens is unnecessarily wide. The use of CH₅₀ units merely obscures these difficulties.

Table 2 Variation of activity ratios with three batches of sheep red cells, expressed as a percentage

	Sheep number		
	7	8	9
Human serum	100	100	100
Joint fluid A	75	85	71
Joint fluid B	35	32	33

The occurrence of normal and abnormal inhibitors of complement lysis in body fluids was discussed by Gewurz and Suyehira (1976). We have found that plotting percentage haemolysis against sample volume using the mathematical transformation of Kabat and Mayer (1961) for assay results did not always give parallel straight lines. Such parallelism is a necessary condition for an assay to be valid and accurate (Finney, 1952). The likelihood that the human agglutinins, rheumatoid factors, and immune complexes present in some specimens were interfering was considered. A sheep was selected, the cells of which were not agglutinated by a number of human sera, including one reference anti-A serum, following a suggestion of Lachmann and co-workers (1973). Repeated assays over two months upon a single serum gave a smaller range of values for serum volume giving 50% lysis when blood from this sheep was used (Table 4), compared to earlier results from random bleedings (Table 1). Two assays done six days apart with the same sheep cells gave results which agreed well (Table 5). Somewhat greater differences appeared in an assay in which two batches of blood from this sheep were compared (Table 6). By choosing suitable animals as a source of cells it seems probable that better intra- and inter-laboratory agreement between 'complement' assays may be achieved. Further improvement in the assay

Table 4 Variation of sample volume required for 50% lysis with different cell batches from one sheep

	Assay date					
	1 Jul	1 Aug	19 Aug	25 Aug	25 Aug	14 Sept
Volume (μl) of ref. sample (789/77)	32	25.5	31	32	36	36

Table 5 Effect of storing cells upon assay values of complement activity

Specimen	Cells batch 1 7 days old	Cells batch 1 13 days old
926 (JF)	62 (37)	65 (37)
943 (B)	68 (34)	65 (37)
955 (JF)	14 (165)*	11 (217)*
966 (JF)	55 (42)	50 (48)
789 (B)		
ref. sample	75 (32)	75 (32)

*Values obtained by extrapolation. Specimens of blood serum (B) or joint fluid (JF), as indicated. Results given as % of the mean activity of normal sera (volumes in μl in parentheses).

Table 6 Results of one assay using two batches of cells from a selected sheep

Specimen	Cells batch 1 13 days old	Cells batch 2 6 days old
926 (JF)	65 (37)	75 (36)
943 (B)	65 (37)	64 (42)
955 (JF)	11 (217)*	10 (262)*
966 (JF)	50 (48)	51 (53)
995 (B)	126 (19)	117 (23)
789 (B)		
ref. sample	75 (32)	75 (36)

*See footnote to Table 5.

Table 3 Variation of total haemolytic complement activity, expressed as a percentage of a normal serum (EW), with three genetic types of sheep red cells

Series*	Serum†	Type of sheep red cells tested					
		HK (MM)		LK (LM)		LK (LL)	
I		Sheep 2562	Sheep S	Sheep 486	Sheep 107	Sheep 2582	Sheep 183
	EW	100	100	100	100	100	100
	MV	58.6	62.7	56.6	55.2	51.0	52.2
	JV	98.4	98.5	92.9	82.4	95.0	91.8
II		Sheep 68		Sheep 42		Sheep 70	Sheep 27
	EW	100		100		100	100
	PT	60.3		67.3		64.7	63.3

(Values calculated from results of Bell *et al.*, *Journal of Immunology*, 108, 467-474 (1972) with the authors' permission.)

*Series I utilised six Duke University sheep; series II tested four Rochester sheep. Each series is listed separately because data were obtained months apart and utilised different bleedings of EW.

†EW and PT—normal donors; MV—active systemic lupus erythematosus (SLE); JV—inactive SLE.

may be possible if rabbit antiserum containing little or no IgG antibody is used, so that rheumatoid factors cannot interfere.

In summary, therefore, the assay described by Kabat and Mayer (1961) for measuring 'total haemolytic complement' is unsatisfactory in several respects:

1 The CH_{50} unit is defined in terms of cell numbers and takes no account of the large differences in sensitivity to complement lysis of cells from different sheep.

2 Reliable measurements of haemolysis at 541 nm demand good spectrophotometers.

3 An implicit assumption, which is not true, is that human body fluids show only quantitative differences in behaviour in complement assays. Even when appropriate controls and one cell source are used some specimens still have lytic characteristics different from that of a normal serum. At present the anomalies of the 'total haemolytic complement' assay are such that it can be considered semi-quantitative only.

The major difficulty which appears in consequence is that of re-interpreting the published data of 'complement' levels in patients with various diseases, which give the results in the CH_{50} units of Kabat and Mayer (1961) or related units. Only recently has the necessity for internal serum standards for assays become apparent and, even so, assays are not rigidly standardised. The CH_{50} values reported, for example, by Sonozaki and Torisu (1970) in patients with either osteo- or rheumatoid arthritis, cover an unexpectedly wide range and show a considerable overlap. Our results suggest that improvements in the technique for measuring complement activity may enhance the ability of the test to discriminate between patients with different diseases.

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