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Hotting-up the complement-fixation test

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SUMMARY

A detailed investigation into the effect of modifying the incubation temperature of the complement-fixation (CF) test is described. For varicella-zoster virus cytomegalovirus and rubella virus, increasing the incubation temperature progressively increased the sensitivity of the CF test to reach a maximum at 15 °C, at which temperature the geometric mean titre of seropositive samples was significantly greater than that found at 4 °C. For these three viruses, each serum shown to contain IgG antibodies by ultrasensitive radioimmunoassay procedures was detected by CF following incubation at 15 °C. No false-positive reactions occurred at 15 °C, but it was our impression that anticomplementary activity was enhanced at this temperature. Significant increases in antibody titre at 15 °C were also seen when measles virus, respiratory syncytial virus, adenovirus and *Mycoplasma pneumoniae* were employed as CF antigens. The results demonstrate that the CF test should be performed at 15 °C if optimum sensitivity is to be achieved. The ability of the test to detect significant rises in antibody titre was not impaired at the higher incubation temperature.

INTRODUCTION

The complement-fixation (CF) test has become an established part of the diagnostic armamentarium available to virology laboratories. Early descriptions of the technique and standard texts advise that the reagents be incubated overnight at 4–8 °C or, if results are required urgently, for 60–90 min at 37 °C; we can find no reference to intermediate temperatures having been employed (Schubert, Stanford & Tiffany, 1951; Bradstreet & Taylor, 1962; Sever, 1962; Fenner & White, 1976; Grist *et al.* 1979; Hawkes, 1979). A deviation from our standard laboratory protocol of incubating the assay overnight at 4 °C has prompted an investigation into alternative incubation temperatures.

A new member of our laboratory staff was being taught the CF test by performing assays on sera in parallel with an experienced worker. On one occasion the novice failed to refrigerate the plastic microplates containing sera, various CF antigens and complement but instead left them on the laboratory bench overnight. When his mistake was discovered the next day we expected the assay to have been ruined but, nevertheless, the test was proceeded with. Much to our surprise the test not only appeared to work correctly following incubation at room temperature

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but, when the results were compared to the duplicate samples which had been held at 4 °C overnight, the serum antibody titres were, in virtually every case, one or two doubling-dilutions higher. This observation stimulated us to vary the incubation temperature under controlled conditions and the results are presented here in detail.

MATERIALS AND METHODS

Serum samples

Sera from 44 healthy female nurses previously tested by radioimmunoassay (RIA) for the presence of IgG class antibodies against varicella-zoster virus (VZV) were available for study (Campbell-Benzie, Kangro & Heath, 1981).

Sera from 80 pregnant women who booked for antenatal care consecutively during October and November 1981 were collected as part of a study of cytomegalovirus (CMV) infections during pregnancy (Griffiths, Campbell-Benzie & Heath, 1980). They were tested by RIA at serum dilutions of 1 in 400 and 1 in 1600 for the presence of IgG antibodies against CMV by a method previously described (Kangro, 1980). Sixty of these sera were also tested for the presence of CF antibodies against measles virus, respiratory syncytial virus, adenovirus and *Mycoplasma pneumoniae*.

For the measurement of antibodies against rubella virus, sera from 21 of the pregnant women described above were randomized with 19 similar sera known not to contain rubella-specific antibodies detectable by single radial haemolysis (Morgan-Capner *et al.* 1979).

Paired sera from 18 patients known to have significant (four-fold or greater) rises in antibody titre were selected from those submitted to our diagnostic laboratory. Eight patients had significant increases in antibody titre against herpes simplex virus, five against CMV, two against *Mycoplasma pneumoniae*, two against respiratory syncytial virus and one against adenovirus.

Complement fixation test

The CF test was performed in microplates as previously described (Griffiths, Buie & Heath, 1978). Briefly, two CF units of antigen, complement (Tissue Culture Services Ltd) and haemolysin (Wellcome Reagents Ltd) were used. Sheep erythrocytes were obtained from Tissue Culture Services Ltd. The end-point of each titration was taken as the reciprocal of the highest dilution of serum showing no haemolysis. Doubling dilutions of test sera were prepared from an initial serum dilution of 1 in 4 and samples possessing CF activity at this dilution were considered positive.

Some virus antigens and control antigens were obtained from Hoechst Pharmaceuticals (VZV, CMV) or Wellcome Reagents Ltd (rubella) while the remainder were kindly provided by the Public Health Laboratory Service.

The overnight incubations were performed in a cooled CO_2 incubator (Gallenkamp IH-290). Variations in temperatures were monitored with maximum–minimum thermometers and all readings were found to be within 0·5 °C of the stated value.

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Table 1. *The effects of varying incubation temperatures on the sensitivity and specificity of the complement-fixation test used to detect antibody against varicella-zoster virus in sera from* 44 *nurses*

	No. of sera giving stated result for IgG antibody by					
		CF with an incubation temperature (°C) of				
	RIA	4	10	13	15	17*
Positive	35	26	27	32	35	28
Negative	9	18	17	12	9	7

* Nine sera produced anticomplementary reactions.

Table 2. *The effects of varying incubation temperatures on the sensitivity and specificity of the complement-fixation test used to detect antibodies against cytomegalovirus in sera from* 80 *pregnant women*

	No. of sera giving stated result for IgG antibody by			
		CF with an incubation temperature (°C) of		
	RIA	4	10	15
Positive	45	41	43	45
Negative	35	39	37	35

Statistical analysis

The significance of the observed difference between geometric mean titres (GMTs) of CF antibodies was assessed by t-test (Armitage, 1971). All titres were expressed in $\log_2$ units.

RESULTS

Preliminary experiments at overnight incubation temperatures of 4, 15, 20 and 25 °C showed that no lysis of sensitized erythrocytes occurred, in test or control wells, at the two highest temperatures, probably as a result of complete inactivation of complement. Experiments were therefore subsequently performed in detail at a range of temperatures below 20 °C.

Assay of antibodies against varicella-zoster virus

The results of testing 44 sera by CF at various incubation temperatures, and by RIA, are shown in Table 1. The standard CF method, employing incubation at 4 °C, detected only 26 (74 %) of the 35 sera which had VZV antibodies detectable by RIA. The ability to detect seropositive samples by CF increased progressively as the incubation temperature was raised until, at 15 °C, all 35 RIA-positive samples had detectable CF antibody. Increasing the incubation temperature to 17 °C resulted in an unacceptably high number of sera giving anticomplementary

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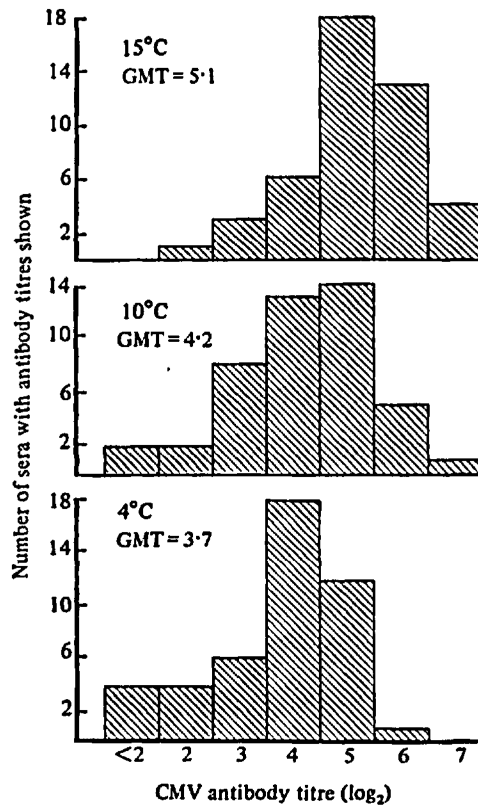

Fig. 1. Distribution of complement-fixing antibody titres found following incubation at three temperatures in sera from 45 pregnant women known to possess IgG antibodies against cytomegalovirus.

reactions. No false-positive reactions were seen at any temperature, showing that the increased ability to detect relatively small amounts of antibody was specific for the virus antigen being employed.

The relative improvements in the sensitivity of the CF test were quantitated by comparing the antibody titres found at each incubation temperature in the group of 35 sera shown, by RIA, to contain antibodies against VZV. The GMT increased only marginally when the incubation temperature was raised to 10 °C. However, the GMT found at 13 °C was significantly ($P < 0·01$) greater than that found at 4 °C. A further significant ($P < 0·05$) enhancement of the GMT occurred between 13 °C and 15 °C so that the overall increase in sensitivity from a GMT of 3·1 at 4 °C to 5·2 at 15 °C was statistically highly significant ($P < 0·001$).

Assay of antibodies against cytomegalovirus

The standard CF method identified 91 % (41 of 45) of the sera shown, by RIA, to possess antibodies against CMV (Table 2). Two of the four discordant samples were detected by CF when incubation was carried out at 10 °C and all four were detected at 15 °C. All 35 sera lacking antibodies detectable by RIA gave negative reactions at all incubation temperatures, confirming the high specificity of the CF

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Table 3. *The effects of varying incubation temperature on the sensitivity and specificity of the complement-fixation test used to detect antibodies against rubella virus in sera from 40 pregnant women*

	No. of sera giving stated result for IgG antibody by			
		CF with an incubation temperature (°C) of		
	SRH	4	10	15
Positive	21	19*	21*	22*
Negative	19	21	19	18

* One SRH-negative serum reacted in the CF test at all three incubation temperatures.

Table 4. *Results obtained at two incubation temperatures when four microbial antigens were employed in the complement-fixation test*

Microbial antigen employed	No. of sera reacting at any temperature	Geometric mean titres			Significance of difference
		at 4 °C	at 15 °C	difference	($P <$)
Measles virus	48	2·98	3·58	0·60	0·05
Respiratory syncytial virus	44	2·00	2·66	0·66	0·01
Adenovirus	43	2·16	2·91	0·75	0·001
Mycoplasma pneumoniae	22	1·27	2·09	0·82	0·001

test. Two sera exhibited partial fixation at dilutions of 1 in 4, which was also observed in the buffer control and antigen control wells. This anticomplementary activity was readily identified as such, but was definitely more pronounced in those tests incubated at the two highest temperatures.

Figure 1 shows that the GMT of the group of 45 RIA seropositive samples increased from 3·7 at 4 °C to 5·1 at 15 °C; a highly significant ($P < 0{\cdot}001$) difference.

Assay of antibodies against rubella virus

When the CF test was performed at 4 °C, 18 (86 %) of the 21 sera possessing rubella-specific antibodies detectable by SRH were identified while at 15 °C all 21 gave positive CF reactions. However, as shown in Table 3 one serum which was persistently SRH negative reacted in the CF test at all three incubation temperatures.

The CF GMT of the 21 SRH-positive samples was higher (3·4) at 15 than at 4 °C (2·9) but this difference did not reach statistical significance ($P < 0{\cdot}10$).

Assay of antibodies against other infectious agents

Having ascertained that incubation at 15 °C provided optimum results for VZV, CMV and rubella it was decided to compare the effects of performing the CF test at 4 and 15 °C when other microbial antigens were employed. The results in Table 4 demonstrate that, for each of the four antigens used, significant increases in GMTs were found when the CF test was incubated at 15 °C.

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Examination of serial sera showing significant increases in antibody titre

To ensure that performing the CF test at 15 °C would not have a differential boosting effect on low titres of antibody such that apparent differences between paired sera would be reduced, serial samples with significant increases in antibody titre were examined. As detailed in Materials and Methods, 18 pairs of sera with four-fold or greater increases in antibody titre were available. When titrated in parallel at 4 and 15 °C, generally increased titres were seen at the higher temperature but, in all cases, both low and high titres were boosted equally so that the four-fold differences detected at 4 °C were still apparent at 15 °C.

Chessboard titrations

In order to define which reagents in the CF test accounted for the observed increase in sensitivity at 15 °C, multiple chessboard titrations were performed in parallel at 4 and at 15 °C. Haemolysin/complement chessboard titrations gave identical patterns at both temperatures. To ensure that incubation at 15 °C was not affecting the sensitivity of the assay indirectly by partially inactivating the complement, the following experiment was also performed. Freshly reconstituted complement, diluted 1 in 10, was divided into three portions and held overnight at 4, 15 and 20 °C. Doubling dilutions of each portion were then made in microplates starting from 1 in 2 and 1 in 3 dilutions, and sensitized erythrocytes were added. Identical patterns of lysis were found in the 4 and 15 °C titrations, showing that overnight incubation at 15 °C had not affected the potency of the complement. In contrast, the titre of the diluted complement after overnight incubation at 20 °C was between 3 and 4, compared to a titre of between 16 and 24 at 4 °C. A parallel experiment starting with a 1 in 40 dilution of reconstituted complement showed titres of between 6 and 8 at both 4 and 15 °C but < 2 at 20 °C. These results confirmed that complement was readily inactivated at an incubation temperature of 20 °C.

Antigen/antiserum chessboard titrations for VZV produced an optimum titre of 64 for both antigen and antiserum at 4 °C. At 15 °C, the optimum antigen titre was again 64 but the antiserum titre had increased to 256. For rubella, the corresponding chessboard titrations revealed optimum titres of 32 and 16 for antigen and antiserum respectively at 4 °C and 32 and 32 at 15 °C. For CMV, the optimum titres were 8 and 32 at 4 °C and 16 and 64 at 15 °C. Antigen/antiserum chessboard titrations were subsequently performed at 4 and 15 °C for the four CF antigens listed in Table 4. The adenovirus titration end-points were identical at 4 and 15 °C. For two viruses (measles and respiratory syncytial) a twofold increase in both antiserum and antigen titres was seen at 15 °C whereas, for *Mycoplasma pneumoniae*, only the antiserum titre increased. We take these results to indicate that an increase in incubation temperature predominantly affects the efficiency with which antibody binds to antigen since, overall, the increase in antiserum titre was more pronounced than the increase in the titre of antigen.

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DISCUSSION

By serendipity we learned that variations in incubation temperature could markedly affect the sensitivity of the CF test. Subsequent detailed experiments, the results of which are presented here, have shown that the sensitivity of the assay increases progressively with increasing temperature to reach a maximum at 15 °C. Attempts to enhance the assay, by increasing the temperature further, resulted in virtually complete inactivation of the complement used.

We interpret these results as indicating that suboptimal quantities of specific antibody are bound to virus antigen at 4 °C and that further molecules of antibody can be persuaded to take part in this reaction by increasing the incubation temperature. This interpretation, rather than a possible effect of temperature on some of the other reagents used, is supported by the results of performing parallel chessboard titrations at 4 and 15 °C. The complement/haemolysin chessboard titrations gave identical results at both temperatures, showing that these reagents had not been affected. The antigen/antiserum chessboards at 15 °C generally showed a more marked enhancement in antiserum titre than in antigen titre. Our conclusion that this represents increased antibody activity at the higher temperature is supported by the results of Cameron (1978) who showed that elevated incubation temperatures were required if a radioimmunoassay for HBsAg was to produce results within a few hours.

For three viruses (VZV, CMV, rubella) reference tests were available which permitted the sensitivity and specificity of the CF test to be evaluated. For VZV and CMV all sera reacting in the 15 °C CF test also contained antibodies detectable by RIA. For rubella, one serum lacking antibodies detectable by single radial haemolysis reacted in the CF test, but this sample has since been shown to contain rubella-specific antibodies by a radioimmunoassay procedure (kindly tested by Dr Mary Anderson). Thus, we believe that, rather than giving a false-positive CF reaction this single serum sample was giving a false-negative SRH result. Therefore, we conclude that the specificity of the CF test is not impaired by incubation at higher temperatures.

We also chose to determine the effects of increasing CF incubation temperature for infectious agents for which we do not possess reference tests. When four commonly employed microbial CF antigens were used, statistically significant increases in GMTs were noted when the CF test was performed at 15 °C. Although we cannot rigorously exclude the possibility that these reactions were false-positives, the most likely explanation is that they resulted from an enhanced ability to detect low levels of antibody specific for each agent. We therefore conclude that optimum results for most, if not all, antigens will be obtained when the CF test is incubated at 15 °C. Since the results of testing 18 pairs of sera demonstrated that significant increases in antibody titre were readily detected at the higher temperature, we see no reason why the routine performance of CF tests in virology diagnostic laboratories should not incorporate overnight incubation at 15 °C. Indeed, this improvement in the CF test could be most useful for those laboratories which do not possess immunoassays for the detection of small amounts of serum antibody.

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