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The Effect of Temperature on the Reactivity of Guinea-Pig Complement with γG and γM Haemolytic Antibodies

M. M. Frank and Thelma Gaither

Department of Health, Education, and Welfare, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation, Bethesda, Maryland 20014, *U.S.A.*

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INTRODUCTION

In the simplest view, complement-mediated cytotoxicity is the result of complement fixation at a cell surface. Several studies indicate that γG antibodies are maximally effective at fixing complement at 4° while γM antibodies are maximally effective at 37°. We have been interested in determining the effect of temperature on complement-mediated cell damage by these two classes of immunoglobulin. This study has had two related objectives. The first object was to define quantitatively those steps in the complement sequence where γG and γM antibodies differ in their interaction with complement components, with an ultimate goal of establishing more useful complement fixation tests. The second objective was to determine whether γM and γG antibodies differ in the mechanism by which they interact with complement components after the initiation of complement fixation. In this report we document the marked differences which exist between the activities of γM and γG haemolysins at 4°. These differences persist in the reactions of the sites SAC1, SAC14, SAC142 and SAC1423. Each of these sites reacts differently depending on the class of antibody which initiated the reaction sequence.

METHODS

Standard methods for preparation of veronal buffered saline with divalent cations and gelatin (VBS), sheep erythrocyte solutions, cellular intermediates in the complement sequence and partially purified guinea-pig complement components were employed (Mayer, 1961; Borsos and Rapp, 1967). Purified human C3 prepared by the Cordis Corporation was obtained through the courtesy of the transplantation immunology branch, NIAID.

A healthy New Zealand white rabbit was immunized by repeated intravenous injection of increasing quantities of boiled washed sheep erythrocyte stromata over a 2½ week period (Mayer, 1961). On the 4th and 5th day following the last injection of antigen the animal was bled. On the 6th day after the last intravenous injection of antigen, the rabbit was injected in each foot pad with 0·2 ml of an emulsion containing equal volumes of antigen and Freund's complete adjuvant; 6 weeks later the animal was bled. Antisera obtained from early and late bleedings from the same rabbit were each separated by Sephadex G-200 gel filtration (Rapp and Borsos, 1966) and a number of the chromatographic fractions were titrated for haemolytic activity (Mayer, 1961). More than 95 per cent of the total haemolytic activity from the initial bleedings was contained in the first peak

(γM) and this peak was further purified as the source of IgM antibody. About 60 per cent of the total haemolytic activity of antisera from the late bleedings was present in the first Sephadex peak and about 40 per cent in the second peak (γG). Purified fractions from the late bleeding were therefore used as the source of γG haemolysin. The separated 19S and 7S peaks were concentrated by ultrafiltration (Diaflo apparatus, Amicon Corporation). The γM was further purified by sucrose density gradient ultracentrifugation (Frank and Humphrey, 1968), and the γG was purified by a second passage through Sephadex G-200. The γM and γG immunoglobulin pools each showed a single line on immunoelectrophoretic analysis. A single molecule of the γM haemolysin used in these experiments was sufficient for C1 fixation, while two molecules of the γG haemolysin were required (Borsos and Rapp, 1965). Optimal antibody concentrations for maximal haemolysis at 37° were determined (Mayer, 1961). Titration of the two antibody pools, performed by the $C\bar{1}$ fixation and transfer method, yielded values of $6{\cdot}6\times 10^{10}$ C1 fixing units/ml at 1 : 500 dilution of γM haemolysin and $2{\cdot}9\times 10^{11}$ C1 fixing units at 1 : 100 dilution of γG haemolysin.

A standard system was used in cold haemolysis experiments in which complement concentration limited the extent of haemolysis. 5 ml of ice-cold VBS was added to 0·5 ml of pre-chilled sensitized sheep erythrocytes or cellular intermediates in the complement sequence, containing $1{\cdot}5\times 10^{8}$ cells/ml VBS. Complement, 0·5 ml of a 1 : 160 dilution in ice-cold VBS, was then added to the mixture. The tubes were placed on a shaking rack in a cold room at 4° and shaken for 15 hours. The cells were sedimented at 0° in an International refrigerated centrifuge, and the amount of haemolysis in the supernatant fluid was determined at 412 mμ in a Gilford Model 240 spectrophotometer. In some experiments in which lysis of EAC142 at 4° was studied, EDTA buffer (Mayer, 1961) rather than VBS was employed so as to block the activities of all but the late acting complement components. Controls consisted of sensitized cells or cellular complement intermediates incubated in buffer at 4° in the absence of complement and unsensitized cells incubated in buffer at 4° in the presence of complement. In no case did the degree of haemolysis of control samples exceed 5 per cent. In every case cellular complement intermediates were tested to be certain that adequate numbers of sites were present. For preparation of EAC1, optimally sensitized cells were offered 20,000 effective C1 molecules per cell to generate a vast excess of SAC1. EAC 142 had at least 100 sites per cell at the start of the period of incubation. EAC 14 preparations had T_{max} times of less than 5 minutes.

Control experiments were performed to determine whether complement inhibitors in serum might be selectively destroying the γM cellular intermediates in the cold and thus preventing lysis of γM sensitized cells. In these experiments, the reaction mixture of EAC 142 suspended in either EDTA buffer or EDTA buffer with added complement was incubated at 4° for 16 hours. The cells were sedimented at 0°, the degree of haemolysis in the supernatant fluid determined, and the button of unlysed erythrocytes resuspended in complement diluted 1 : 50 in EDTA buffer EDTA-C. The mixtures containing the cell button in EDTA-C were reincubated at 37° for 1 hour to determine the number of SAC142 which had survived 16 hours of incubation in EDTA-C or EDTA buffer at 4°.

In kinetic experiments, 0·5 ml samples were taken from reaction mixtures prepared as above at various timed intervals and mixed with 2·5 ml ice-cold EDTA buffer. The mixtures were immediately centrifuged at 800 ***g*** for 5 minutes at 0°, and the extent of haemolysis determined.

Lysis of cells at 4° was also examined in a series of experiments in which antibody concentration rather than complement concentration limited the extent of haemolysis.

Complement was present in relative excess. In these experiments, erythrocyte suspensions containing $0{\cdot}75 \times 10^8$ cells in 0·5 ml of VBS were mixed with 0·5 ml of the various dilutions of antibody. The tubes were incubated with occasional mixing at 37° for 40 minutes and then allowed to proceed at either 4° or 37°. Pre-chilled or pre-warmed compliment, 1 ml of a 1: 50 dilution in UBS, was added. Appropriate controls consisting of cells plus complement in the absence of antibody and cells plus antibody in the absence of complement were included. A 1 : 50 dilution of C represents 20 CH50 units in this test system since a 1 : 1000 dilution of the complement solution produced about 50 per cent haemolysis of optimally sensitized erythrocytes at 37°.

RESULTS

QUANTITY OF ANTIBODY REQUIRED FOR OPTIMUM HAEMOLYSIS AT 37° AND 4°

Baseline studies were performed at 37° to compare activities of the various antibody preparations. The effect of varying concentrations of γG and γM haemolysins on the extent of haemolysis at 37° is shown in Fig. 1. In the case of γM haemolysin the antibody optimum was quite broad and there was no zone of inhibition of haemolysis by excess IgM haemolysin. When γG antibody was employed, the degree of haemolysis increased with increasing concentrations of antibody to a maximum value and then decreased. Thus, greater than optimal amounts of γG antibody led to inhibition of haemolysis.

The effect of a 100-fold variation of antibody concentration on the extent of haemolysis at 4° is also shown in Fig. 1. Haemolysis was allowed to proceed for 15 hours to reach an end point. γM haemolysin produced minimal lysis in the cold even when present in large amounts and an optimum quantity of haemolysin was never reached. Antibody optima for γG haemolysin were similar at 4° and 37°; however, there was no zone of inhibition noted at 4°.

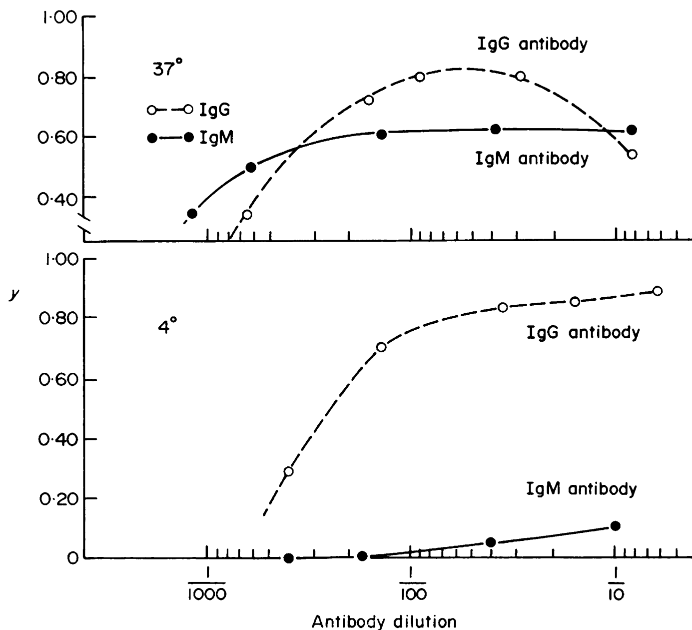

FIG. 1. Lysis of sheep erythrocytes at 37° and at 4° as a function of IgM or IgG haemolysin concentration.

KINETICS OF HAEMOLYSIS BY γM AND γG HAEMOLYSIN AT 4° AS COMPARED WITH 37°

γM antibody produced virtually no haemolysis in the cold over a sixteen-fold range of antibody concentrations, while γG haemolysin produced extensive lysis at both temperatures, although it was somewhat less efficient at 4° than at 37° (Fig. 2). The γG mediated reaction reached a plateau after about 14–16 hours. For end-point analysis experiments,

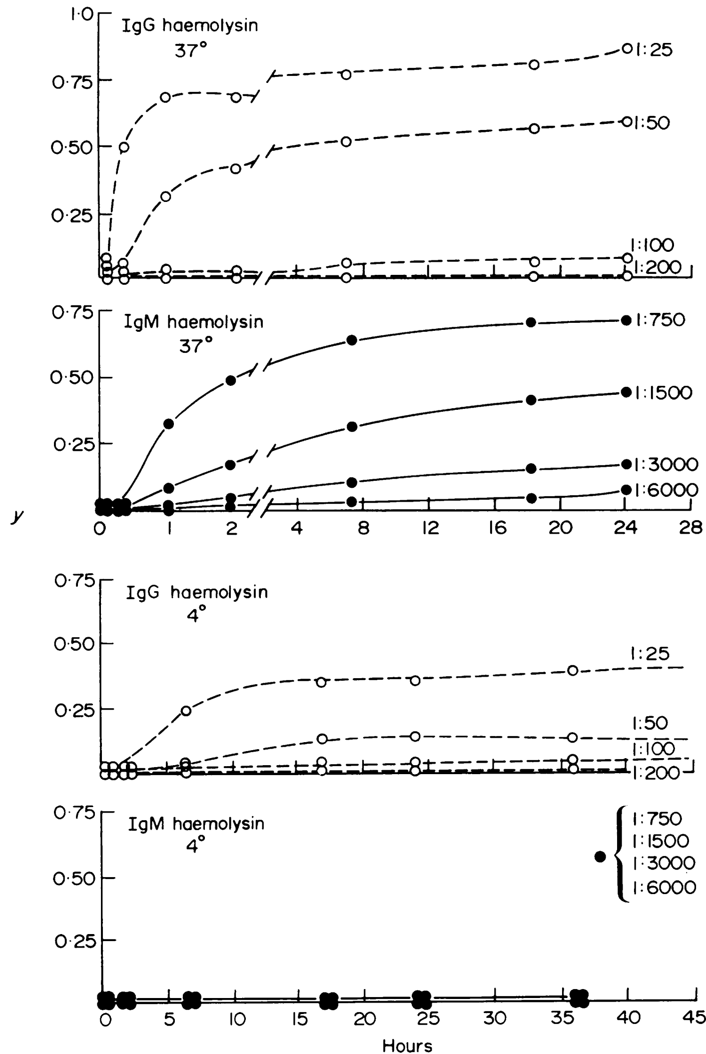

FIG. 2. Kinetics of lysis at 37° and at 4° at various dilutions of antibody.

15 hours was chosen as a convenient length of time for incubation of the reaction mixtures. In several experiments, the reaction was sampled at 15 hours and again at 22 hours, and no significant difference in the degree of haemolysis was found. There was no evidence of γM mediated haemolysis even after 48 hours at 4° (Fig. 2).

LYSIS OF SHEEP ERYTHROCYTES AT 4° AND 37° WHEN ANTIBODY CONCENTRATIONS LIMITS THE EXTENT OF HAEMOLYSIS

The extent of haemolysis in a reaction mixture with limited haemolytic antibody and a large excess of complement is presented in Table 1. The findings of depressed lytic activity of IgM at 4° are similar to those noted in experiments where complement limited the extent of haemolysis. A quantity of γM haemolysin sufficient to produce 100 per cent haemolysis at 37° was noted to be ineffective at lysing cells at 4°, even in the presence of 20 CH50 units of complement, while γG haemolysin was quite efficient under the same conditions.

TABLE 1

LYSIS OF SHEEP ERYTHROCYTES: ANTIBODY CONCENTRATION LIMITING; COMPLEMENT IN EXCESS

	Haemolysin dilution	Haemolysis at 37° (per cent)	Haemolysis at 4° (per cent)
γM	1 : 2,500	—	8·1
	1 : 5,000	100	0
	1 : 10,000	74·3	0
γG	1 : 2,500	100	93·3
	1 : 5,000	92·7	50·7
	1 : 10,000	80·6	15·0

LYSIS OF CELLULAR INTERMEDIATES IN THE COMPLEMENT SEQUENCE AT 4°

The extent of haemolysis in reaction mixtures where various intermediates in the complement sequence were added to whole serum complement and the reaction allowed to proceed at 4° for 15 hours are presented in Table 2. Marked differences in haemolysis are quite evident between γM and γG sensitized erythrocytes with each of the cellular intermediates. The γG haemolysin sensitized erythrocytes lysed much more extensively than the γM sensitized erythrocytes. There was some breakthrough with the intermediates $SA_{\gamma M}C142$ and $SA_{\gamma M}C1423$.

TABLE 2

COMPLEMENT DEPENDENT LYSIS OF γM AND γG HAEMOLYSIN SENSITIZED CELLS AND CELL-COMPLEMENT INTERMEDIATES AT 4° FOR 16 HOURS

	Percentage of γM sensitized cells	Percentage of γG sensitized cells
EA	0·4	32·9
EAC1	1·4	31·8
EAC4	2·6	24·3
EAC14	1·5	13·8
EAC142	18·3	56·6
EAC1423	9·8	45·0
(both cells 4+ immune adherence)		
EAC 1423567	87 (92 at 37°)	100 at 4° and 37°

Control experiments not detailed here demonstrate that $C\bar{1}$ was still associated with the $SA_{\gamma M}C1$ after overnight incubation in the cold and that $C\bar{1}$ dissociated from $SA_{\gamma M}C1$ did not destroy the available fluid phase complement. The results above are contrasted with those obtained with EAC1423567 prepared with γM antibody which lysed as exensively at 4° as at 37°.

Experiments in which EDTA-C provided late-acting complement components for EAC142 haemolysis gave essentially the same results as those in which EAC142 were incubated with whole complement in VBS. Cells sensitized with γG haemolysin lysed more extensively than those sensitized with γM. The γM intermediate lysed to about the same extent in the presence of EDTA-C and in the presence of whole complement. It is interesting that sheep erythrocytes sensitized with haemolysin alone, and cellular intermediates in the complement sequence with antibody and complement on the cell surface, lysed to about the same extent in the presence of whole complement. This finding was true for EAC142 for example which had a T_{max} of less than 2 minutes indicating large numbers of SAC14 per cell.

Table 3

Test for persistance of EAC142 after exposure to whole guinea-pig serum or EDTA buffer for 16 hours at 4°, optimally and suboptimally sensitized cell intermediates employed

	γM sensitized cells		γG sensitized cells	
A. Cells exposed to fresh serum				
Antibody dilution	1 : 40	1 : 640	1 : 45	1 : 1215
Lysis after 16 hours at 4° (per cent)	14·2	1·2	39·4	4·5
Lysis on resuspending EDTA-C at 37° (per cent)	92·3	11·5	87·0	38·1
B. Cells exposed to EDTA buffer				
Antibody dilution	1 : 40	1 : 640	1 : 45	1 : 1215
Lysis after 16 hours at 4°	0	0	0	0
Lysis on resuspending in EDTA-C at 37° (per cent)	100	32	100	77

Table 3 shows results of experiments designed to test the possibility that an inhibitor of complement components, active in the cold, was destroying cell bound γM-complement sites or making them nonreactive. EAC142 were incubated at 4° in the presence of whole serum complement. The degree of haemolysis was of the same order of magnitude as that shown in Fig. 2. The washed cells from these reaction mixtures lysed extensively on being exposed at 37° to the late acting components of complement demonstrating the persistence of sites in the state SAC142, even after 16 hours of exposure to whole serum. EAC142 incubated overnight in the presence of EDTA-C did lyse to a slightly lesser extent than cells allowed to incubate overnight at 4° in the presence of EDTA buffer alone, suggesting that serum is capable of destroying some SAC142. The degree of site destruction was clearly not sufficient to account for the failure of $EA_{\gamma M}C142$ to lyse at 4°.

DISCUSSION

Our results indicate that rabbit γM haemolysins are far less efficient at lysing sheep erythrocytes in the cold than are γG haemolysins over a broad range of antibody concentrations. The marked difference between lytic activities of γM and γG haemolysins is not due to differences in reaction rates, since cells sensitized with both types of antibody were shown in kinetic experiments to reach a haemolytic end point within the 15 hour period of the experimental reaction. Moreover, the large differences are seen when the

extent of haemolysis is limited by antibody concentration as well as by complement concentration. They confirm, quantitate and extend the observation of Colten, Borsos and Rapp (1967) of increased lysis of erythrocytes by γG haemolysin. In addition, these findings in the haemolytic system confirm results of Stollar and Sandberg (1966) that γG antibodies fix complement more readily than γM antibodies when reactions are allowed to take place in the cold.

Increasing the concentration of γG haemolysin beyond the optimal level leads to inhibition of haemolysis in studies performed at 37°, although not at 4° and the plateau region of optimal haemolysis is less broad than that noted with IgM haemolysin. Inhibition of cytocidal activity has been reported previously in bacterial and other systems but to our knowledge has not been reported before in the anti-Forssman system (quoted by Muschel, 1965). Relatively few γM haemolysin molecules are required to sensitize a cell while thousands of γG molecules are required. The inhibition of lysis by excess γG haemolysin might be accounted for, if increasing the number of haemolysin molecules beyond the optimal level leads to steric interference with the complement proteins.

Cellular intermediates prepared with the two classes of haemolysin also differ in their ability to lyse cells in the cold in the presence of complement. Marked differences were noted with cell-complement intermediates EAC1, EAC4, EAC14, EAC142 and EAC1423, although the temperature differential was least marked with EAC1423. EAC1423567 prepared with γM haemolysin lysed equally well in the warm and in the cold. It has been shown (Colten *et al.*, 1967) that γG haemolysin fixes C1 in the cold better than does γM haemolysin and it might be expected that intermediates beyond this step in the haemolytic sequence would be independent of the marked temperature dependence of complement fixation. As noted above, this is not the case.

Our studies suggest that temperature dependent, inhibition of lysis by complement of γM sensitized cells, takes place at more than one step in the haemolytic sequence. The studies presented here indicate that the point of inhibition must be prior to the formation of EAC1423567. There is evidence that inhibition is noted prior to and after the formation of EAC142 for we find that γM haemolysin sensitized erythrocytes are less efficient at EAC142 formation at 4° than at γG sensitized erythrocytes as well as less efficient at proceeding to lysis. Investigations of these phenomena are complicated by the differing efficiencies of complement components with each class of antibody, even at the same temperature (Frank and Gaither, 1970). Further work is required to define precisely the points of inhibition.

It is of interest that γG cellular intermediates in the complement sequence do not lyse to a much greater extent at 4° in the presence of whole serum complement than do cells sensitized by antibody alone. This finding probably indicates that the number of one, four, two and perhaps three sites generated per cell does not limit the extent of haemolysis in the reaction of whole serum complement with sheep erythrocytes sensitized by rabbit haemolysin. If the generation of these sites limited the extent of haemolysis when whole serum reacts with sensitized cells, prior formations of these sites on cell surfaces might have been expected to increase the degree and rate of haemolysis. We have demonstrated that observed differences in the activities of EAC142 prepared with γM and γG antibodies are not simply due to serum inhibitors of cellular intermediates which destroy the sites in the cold, unless these inhibitors no longer influence complement components when the mixture is warmed. The data presented here lend additional support to the concept that cells sensitized with γG and γM antibodies interact with complement through different mechanisms.

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