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# Evidence that Rabbit γG Haemolysin is Capable of Utilizing Guinea-Pig Complement More Efficiently than Rabbit γM Haemolysin

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**Summary.** Sheep erythrocytes sensitized with rabbit γG anti-Forssman haemolysin may be haemolysed more efficiently and to a greater extent by limited amounts of guinea-pig complement components than are cells sensitized with γM anti-Forssman haemolysin. This apparent difference, noted in all components titrations, was two-fold or greater in titrations of C2 and components following C2 in the haemolytic sequence. Neither site to site transfer of C2 nor AC142 could account for the 100 per cent greater haemolytic efficiency of C2 in haemolytic assays. These results indicate that immunoglobulin class plays a more complex role in immune haemolysis than simple initiation of complement fixation and suggest that γG complement fixing sites may be more efficient than γM sites in producing biological damage.

## INTRODUCTION

It is established that antibodies of the immunoglobulin classes γM and γG fix C1 and initiate complement fixation by different mechanisms. A single molecule of γM antibody on a cell surface is sufficient to fix C1 and initiate complement mediated cell damage. Two molecules of the usual serum γG antibody, side by side, a so-called antibody doublet, are required to achieve these same biological effects (Borsos and Rapp, 1965; Humphrey and Dourmashkin, 1965). Differences between γM and γG haemolysins have also been demonstrated in their relative efficiency of C1 fixation at 37° and 4°, in their respective abilities to activate C1 from its inactive precursor form in serum (Colten, Borsos and Rapp, 1967; Colten, Borsos and Rapp, 1969), and in the mechanism by which they fix complement in the presence of particulate as opposed to soluble antigen (Ishizaka, Tada and Ishizaka, 1968).

There is evidence to suggest that these two classes of immunoglobulin differ not only in the mechanism of interaction with C1 but also in the mechanism of interaction with other components of complement (Frank and Gaither, 1970; Frank, Dourmashkin and Humphrey, 1970). In this paper, the apparent efficiency of whole guinea-pig complement and of the complement components C4, C2, C3, C5, C6, C8 and C9, defined as the relative number of molecules required to lyse a sensitized erythrocyte, is shown to be a function of the class of the haemolytic antibody used to sensitize sheep erythrocytes. All of these appear

to be more active in lysing cellular intermediates in the complement sequence prepared with γG antibody and therefore have markedly higher titres when assayed with γG cellular intermediates. This finding may explain many of the discordant results obtained when complement reagents are assayed in different laboratories and account for some of the marked differences in complement component efficiency reported. These results suggest that, although γG antibodies are less efficient at initiating complement fixation than are γM antibodies, they may be more active in terms of biological effectiveness.

## MATERIALS AND METHODS

The sources of sheep erythrocytes and complement, as well as preparation of buffers and partially purified guinea-pig C2, have been previously described (Borsos, Rapp and Cook, 1961 ; Mayer, 1961; Borsos, Rapp and Mayer, 1961). Partially purified C1 was prepared from a pH 5·6 euglobulin precipitate of fresh frozen guinea-pig serum (Borsos and Rapp, 1963). The precipitate was resuspended in veronal buffered saline at pH 7·4 and sedimented in a 10–30 per cent sucrose (w/w) gradient in a preparative ultracentrifuge (Frank and Humphrey, 1968). The macroglobulin peak from the gradient containing the bulk of the C1 activity was pooled as a source of C1. Partially purified C1 was free of guinea-pig anti-sheep erythrocyte antibody. Partially purified C4 and late acting components of complement prepared from guinea-pig serum were obtained through the courtesy of Dr T. Borsos and Dr. H. Shin. They were prepared either by the Cordis Corporation according to the method of Nelson, Jensen, Gigli and Tamura (1966) or by a modification of the Nelson method in the laboratory of Dr M. Mayer (Shin and Mayer, 1968).

Methods for the preparation of immunochemically pure γG and γM immunoglobulin with high titre anti-Forssman activity are presented in the accompanying report. A single molecule of the partially purified γM haemolysin employed in these studies was sufficient to fix $C\bar{1}$ and two molecules of the partially purified γG were required to fix $C\bar{1}$ as shown in the activated C1 fixation and transfer test (Borsos and Rapp, 1965).

The cell complement intermediate, EAC4, was prepared with optimally sensitized cells (Mayer, 1961) as previously described (Borsos and Rapp, 1967). $T_{max}$ of all EAC14 preparations were less than 8 minutes (Mayer, 1961). Cell complement intermediates prepared with γM and γG antibodies were prepared at the same time with the same buffers and the same lots of complement, and all complement titrations were performed with γM and γG intermediates at the same time to minimize variation. Methods for complement component titration and immune adherence have been previously described (Mayer, 1961; Shin and Mayer, 1968; Nishioka and Linscott, 1963; Gewurz, Page, Pickering and Good, 1967; Rommel and Stolfi, 1968).

## RESULTS

The titration of whole complement and partially purified complement components with γM and γG haemolysins and cell-complement intermediates shows that whole complement activity, as well as all complement components activity, is higher with the γG haemolysin sensitized cells (Table 1). Two- to five-fold increases in the number of sites per cell were found in titration of components C2, C3, C5, C6, C8 and C9, and increases of about 20 per cent were noted in titrations of whole complement activity. EDTA-C as a source of the late acting complement components was far more efficient in lysing EAC14 and EAC142

TABLE 1

RELATIVE TITRES OF WHOLE GUINEA-PIG COMPLEMENT AND PARTIALLY PURIFIED COMPONENTS ASSAYED WITH γG AND γM HAEMOLYSIN SENSITIZED ERYTHROCYTES

| | γM | γG |
|---|---|---|
| Whole C($CH_{50}$) | 177 | 201 |
| Cl (Z × dilution) | 7500 | 9500 |
| C2 | 748 | 2380 |
| C3 | 51 | 158 |
| C4* (whole serum) | 4545 | 14285 |
| C4† (whole serum) | 20833 | 25600 |
| C4† (partially purified) | 1220 | 1667 |
| C5 | 1120 | 4600 |
| C6 | 660 | 1470 |
| C8 | 580 | 2240 |
| C9 | 360 | 1724 |
| C3 by immune adherence ($EAC142_{GP}$) | | |
| G.P. C3 | 150 | 1200 |
| Hu. C3 | 20 | 180 |

Adequate reagents were not available for C7 titrations. Titres are relative. Plotting Z vs component concentrations over a broad range does not give a straight line with a slope of 1 in the case of a number of the late acting components.

*Assayed by method described by Gewurz *et al.* (1967).

† Assayed in the presence of EDTA-C optimal amounts for each immunoglobulin.

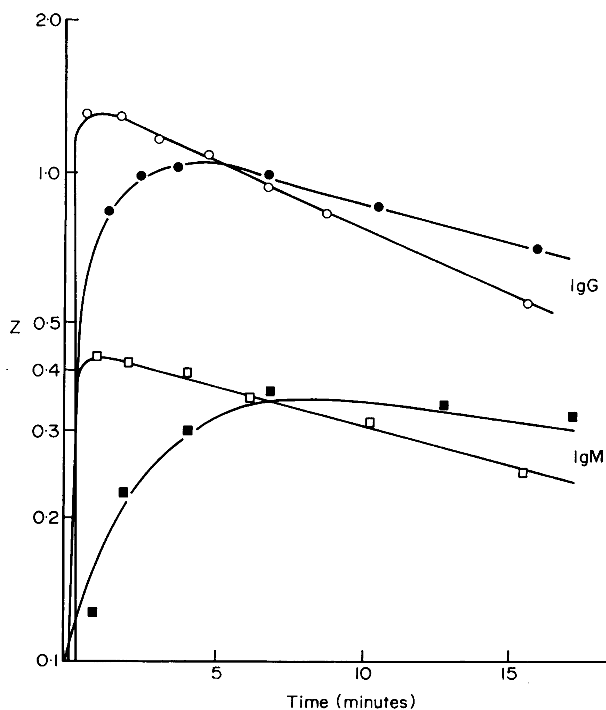


FIG. 1. Kinetic studies of the interaction of $EA_{\gamma M}C14$ and $EA_{\gamma G}C14$ with C2. $Z = -\ln(1-y)$. ○, 1/45 dilution of γG haemolysin; ●, 1/405 dilution of γG haemolysin; □, 1/40 dilution of γM haemolysin; ■, 1/640 dilution of γM haemolysin. The figures are plotted on semilog basis to allow a rough comparison of decay rates.

prepared with γG haemolysin. The marked differences in complement activity were also noted in titrations of C3 by immune adherence.

Kinetic studies of the lysis of EAC14 in the presence of a 1 : 1700 dilution of partially purified guinea-pig C2 are illustrated in Fig. 1. In order to determine whether the differences might be due to differences in relative antibody concentration on cell surfaces, cells were sensitized with optimal amounts of antibody and also with suboptimal amounts of antibody. The difference in the haemolytic efficiency of C2 is obvious at both antibody concentrations.

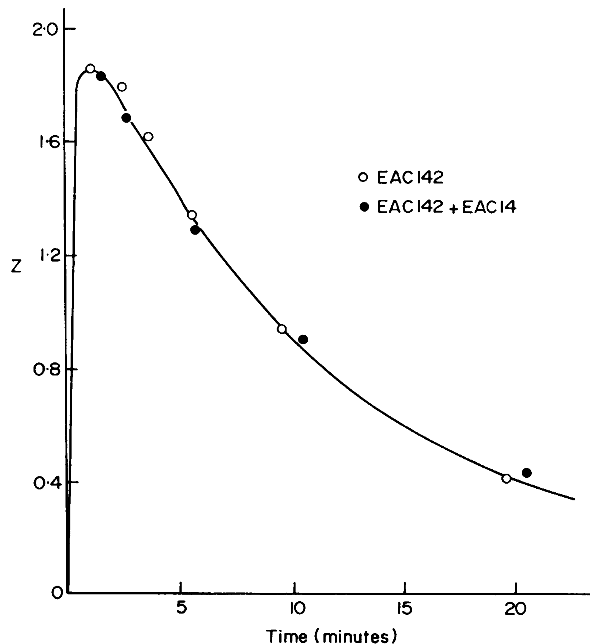


FIG. 2. Kinetic study of the interaction of $EA_{\gamma G}C142$ prepared at 0° and EDTA-C, in the absence of (○) and in the presence of (●) $EA_{\gamma M}C14$ stroma. $Z = -\ln(1-y)$.

It is possible that the two to three-fold increase in the titre of C2 when assayed with $EA_{\gamma G}C14$ was due to the transfer of $A_{\gamma G}C142$ from cell to cell, since transfer is known to occur under exceptional circumstances (Willoughby and Mayer, 1965). To investigate such a possibility, boiled sheep erythrocyte stromata were added to the kinetic $T_{max}$ mixtures. The concentration of stromata was such that there was 100-fold excess of stromal antigen over cellular antigen in the fluid surrounding the cell complement intermediates. Thus, the cell stroma might have acted as a 'sink' for AC142. No transfer of AC142 occurred since the $T_{max}$ and the degree of haemolysis were identical in the control and in the test EAC142 mixtures. A similar experiment in which a large excess of EAC14 stroma was suspended in the fluid surrounding washed EAC142 revealed no differences in $T_{max}$ or extent of lysis suggesting that C2 transfer is not responsible for the differences seen (Fig. 2). Addition of stroma sensitized with IgM haemolysin and C1 to EAC14 prepared with limited purified C4 showed no decrease in C4 titre; therefore, transfer of C4 from site to site could not be demonstrated. Reutilization of C3 in immune adherence would be unlikely as an explanation for the higher titre of C3 with the γG cellular intermediate.

These control experiments (not performed with C5, 6, 8 and 9) suggest that transfer and reutilization of complement components by several different cellular sites is not responsible for the increased titre noted with γG cell intermediates. The higher apparent titre of C4 could be shown to be related to the greater efficiency of EDTA-C in lysing γG sensitized EAC142. This greater efficiency of guinea-pig EDTA-C was also noted in C2 titrations when γG sensitized EAC 14 were used as indicator cells. Nevertheless, even when high concentrations of EDTA-C were employed, twice as many C2 haemolytic sites were generated when γG sensitized EAC14 were compared to γM sensitized EAC14.

## DISCUSSION

These studies demonstrate that the titre of the whole guinea-pig complement, as well as its individual components, may be markedly influenced by the class of immunoglobulin haemolysin which initiates complement fixation and which is used to prepare the cell–complement intermediate complexes. A similar observation has been made in titrations of guinea-pig C1. However, in this case it is thought that site to site transfer is responsible for the elevated C1 titre found with γG haemolysin (Colten *et al.*, 1969). The possibility of site to site transfer of C2 and C4 from cells sensitized with γG antibody has been examined in detail and we are unable to demonstrate sufficient transfer to account for the alteration in the titres of these components. That site to site transfer of C2 and C4 does not occur when sites are prepared with predominantly IgM antibody in unfractionated antisera has previously been shown (Borsos and Rapp, 1963).

The marked increase in titre of the various complement components tested with γG haemolysin–complement–cell intermediates probably reflects increased haemolytic efficiency of several of the components. The evidence for increased efficiency is most convincing for C2 and C3 where an estimate of the number of active cell complement sites with which the test component may react can be made and cells with an equal number of γG and γM complement-intermediate sites can be provided. In addition, we have evidence not detailed here that the conditions used in preparation of $EA_{\gamma M}C4$ and $EA_{\gamma G}C4$ affect component titration at a point in the sequence beyond EAC142. The increase in titre noted with γG haemolysin is much less impressive in whole complement titration than in titration of individual complement components. Whole complement titres varied when titrations were performed with γG haemolysins prepared from four different anti-Forssman antisera. However, in each case γG sensitized cells were lysed more efficiently in the presence of very limited amounts of complement than were γM sensitized cells. The effect of variation in antibody subclass and avidity of antibody in these reactions is under study at present.

Spooner and Sell (1966) have reported that γG anti-Forssman haemolysin is equally efficient in lysing nucleated and non-nucleated cells, while γM haemolysin is relatively inefficient in lysing nucleated cells. Our data suggest that this difference may be due to the ability of γG complement fixing sites to utilize complement more efficiently than γM complement fixing sites and produce more damage to the cell membrane when complement is present in excess. A nucleated cell may be able to repair degrees of membrane damage which might lyse a non-nucleated cell. Studies of the damage to cell membranes produced by antibody and complement as visualized in the electron microscope support this hypothesis (Frank *et al.*, 1970).

A possible explanation of the increased efficiency of the various complement components, noted to be almost exactly two-fold in the case of guinea-pig C2, is that a γG haemolysin doublet may have a two-fold symmetry about the antibody molecules, allowing for haemolytically effective sites on either side of the doublet. IgM haemolysin may not allow for this possibility (Fig. 3). This hypothesis supposes that the antibody plays a role in spatial orientation of complement components to produce a haemolytically effective site.

Fig. 3. Possible mechanism for the greater efficiency of the γG haemolysin doublet utilization of guinea-pig complement. The arms of the Y represent Fab fragments of the immunoglobulin molecule and the body of the Y represents the Fc fragment. C2 is the first component in the haemolytic sequence to show a two-fold greater efficiency in titre when assayed with γG complement–cell intermediates.

The striking differences reported here between IgM and IgG sites may explain some of the variation reported among various laboratories in the efficiency of complement components (Tamura and Nelson, 1967; Müller-Eberhard, Dalmasso and Calcott, 1966). These various laboratories do not use the same source of immunoglobulin, the same class of immunoglobulin and, often, antibody to the same antigenic groups on the cell surface. The results suggest that, although γG antibody is inefficient at initiating complement fixation in that two γG molecules, side by side, are required for initiation of complement fixation, γG may be far more efficient in terms of utilization of complement components once the site is formed and may be expected to produce far more biological damage.

## ACKNOWLEDGMENTS

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