The Assembly of Early Components of Complement on Antibody–Antigen Aggregates and on Antibody-Coated Erythrocytes

By JOHN W. F. GOERS* and RODNEY R. PORTER

M.R.C. Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

(Received 23 March 1978)

Radioimmune assays were developed to assay the binding of complement components C1q, C1s and C4 to antibody aggregates and to cell-bound antibody. The binding of the components was compared with the haemolytic activity and with the capacity to form the C3 convertase activity in the presence of excess C2. The destruction of whole complement and of C4 activity is similar per 1000 molecules of antibody in aggregates and cell-bound antibody, as is the binding of C1q and C1s, the latter being in a 1:2 molar ratio. The binding of C4 is about 12 times greater, per 1000 molecules of antibody, on cells than in aggregates. However, the effective C4 molecules, as judged by the formation of C3 convertase activity, are much more similar on cells and aggregates. An assembly mechanism of the early components of complement on antibody-coated cells, which is compatible with these results, is suggested.

The early components in the classical pathway of complement activation bind to antibody–antigen aggregates or antibody-coated cells, resulting in a multimolecular complex formed from the fourth, second and third components C423. This complex has proteolytic activity and will activate the fifth component, which forms a further complex with the later components C56789 causing the lysis of cells (Müller-Eberhard, 1975; Fothergill & Anderson, 1978; Porter, 1978). The formation of these biologically active complexes appears to be an essential feature of the complement system, but little is known of the molecular basis of these reactions.

The formation of the C423 complex depends on the binding of the first component, C1, which contains three subcomponents C1q, C1r and C1s, to aggregated or cell-bound antibody. Component C1 binds to the second constant domain of IgG antibody via C1q. Two molecules each of C1r and C1s form a tetrameric complex in the presence of Ca^{2+} , which binds to C1q through C1r. The bound C1 complex develops proteolytic activity which is responsible for the activation of C4 and C2. Components C4 and C2 form an active complex C42 (C3 convertase) (Müller-

Eberhard *et al.*, 1967; Polley & Müller-Eberhard, 1968) which activates C3, which in turn associates to give the C423 complex with the capacity to activate C5 (Goldlust *et al.*, 1974; Cooper & Müller-Eberhard, 1970).

In this work we have compared the ability of antibody bound to erythrocytes and of antibody aggregated by a soluble protein antigen first to bind and activate C1 and then in turn to bind C4 and develop C3 convertase activity. We have found that antibody on erythrocytes binds similar amounts of C1 as equal amounts of antibody in aggregates, but results in 10–13-fold more binding of C4 to sensitized cells than to aggregates.

Much of the C4 bound, however, is ineffective in the complement sequence, as judged by the development of C3 convertase activity in the presence of excess C2; about 95% is ineffective when bound to sensitized cells and 50% when bound to antibody aggregates. Indirect evidence suggests that the effective C4 molecules are those bound to the Fab portions either very close to or on the same IgG molecules which are binding C1 on their Fc sections.

Materials and Methods

Materials and buffers

Veronal-buffered saline containing 0.142 M-NaCl, 5 mM-sodium barbital, 0.15 mM- CaCl_2 and 1 mM- MgCl_2 , pH 7.4, was prepared fresh daily from 5-fold concentrated stock. Low-ionic-strength buffer was prepared by diluting veronal-buffered saline with an equal volume of 5% (w/v) glucose. Phosphate-buffered saline contained 0.15 M-NaCl and 0.01 M-

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968). IgG, immunoglobulin gamma; IgM, immunoglobulin macro; Fab, fragment antigen binding; Fc, fragment crystalline from the papain digestion of IgG; (Fab')₂, from pepsin digestion of IgG.

* Present address: Department of Chemistry, University of California, 405 Hilgard Avenue, Los Angeles, CA 90024, U.S.A.

potassium phosphate, pH 7.4. Buffered balanced salt solution was prepared as described by Shortman (1968). Veronal-buffered saline/glucose was used in all experiments unless mentioned otherwise. Sephadex G-200, Sephadex G-50, DEAE-Sephadex A-50, CM-Sephadex C-50, Sepharose 6B and CL-Sephadex 6B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Complement components

Functionally pure human C1 was obtained as a euglobulin precipitate of human serum. One part of serum was mixed for 2 h at 4°C with 4 parts of 5 mM-CaCl₂, pH 7.4. The resultant precipitate was washed once with 0.04 M-sodium acetate/5 mM-CaCl₂, pH 5.5, and then dissolved in 0.2 M-NaCl/5 mM-sodium acetate/5 mM-CaCl₂/0.01 % NaN₃, pH 5.5. Finally, the lipid was removed by centrifugation at 75000g for 30 min. Fractions were stored at -70°C for months. Before adsorption on sensitized erythrocytes or immune aggregates, the C1 was activated fully by dilution with 1 vol. of 5 % (w/v) glucose/0.01 M-Tris/HCl, and the pH adjusted to 7.4 with 1 M-NaOH. After incubation at 30°C for 45 min the C1 was clarified by centrifugation at 1000g for 30 min.

Human C1q and C1s were prepared as described by Reid (1974) and Gigli *et al.* (1976). Both C1q and C1s preparations showed at least 95 % homogeneity on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate.

Guinea-pig C1 was prepared in functionally pure form as an euglobulin precipitate by the method of Nelson *et al.* (1966).

Human C4 was purified as described by Gigli *et al.* (1977). Briefly, the C4 was purified from a pseudoglobulin fraction of human serum by chromatography on DEAE-Sephadex A-50 and Sepharose 6B. After the DEAE-Sephadex chromatography step, C4 was functionally free from C1, C2 and C3 and was used for titration experiments. Chemically homogeneous C4 obtained after Sepharose 6B chromatography was used for immunization and standards in the specific radioimmunoassay for C4.

Human C2 was prepared by a modified procedure of Kerr & Porter (1978). After chromatography of pseudoglobulin on CM-Sephadex C-50 and precipitation by 75 % satd. (NH₄)₂SO₄, the precipitate resulting from 500 ml of serum was dissolved in 0.03 M-sodium phosphate/0.01 % NaN₃, pH 7.8, and dialysed overnight at 4°C against the same buffer. The sample was applied to a column (3.5 cm × 35 cm) of DEAE-Sephadex A-50 equilibrated at 4°C in 0.03 M-sodium phosphate/0.01 % NaN₃, pH 7.8. After a washing with 500 ml of the column buffer, C2 was eluted with 0.10 M-NaCl/0.03 M-sodium phosphate/0.01 % NaN₃, pH 7.8. C2 prepared in this manner was

functionally free of C1, C4 and C3 and was stable for months at -70°C.

Guinea-pig C2 was prepared functionally pure as described by Borsos *et al.* (1961).

Human C3 was obtained by the modified procedure of Nilsson & Müller-Eberhard (1965) and Tack & Prahl (1976). The precipitate formed at pH 5.5 obtained from the C4 purification scheme of Gigli *et al.* (1977) was dissolved in 0.15 M-NaCl/0.01 M-potassium phosphate, pH 8.0. This solution was centrifuged for 30 min at 75000g to remove lipids, and then applied at 4°C to a column (3.7 cm × 35 cm) of DEAE-Sephadex A-50 equilibrated in 0.05 M-potassium phosphate/0.01 % NaN₃, pH 8.0. After a washing with 3 litres of the column buffer, the C3 was eluted with a linear gradient formed from 1.5 litres of 0.05 M-potassium phosphate/5 mM-EDTA/0.01 % NaN₃, pH 8.0, and 1.5 litres of 0.05 M-potassium phosphate/5 mM-EDTA/0.15 M-NaCl/0.01 % NaN₃, pH 8.0. The fractions containing C3 were concentrated by ultrafiltration and dialysed against a buffer containing 0.025 M-potassium phosphate/0.1 M-KCl/0.01 % NaN₃, pH 8.0. The sample was then applied to a column (4.2 cm × 40 cm) of hydroxyapatite (Bio-Gel HTP; Bio-Rad Laboratories, Bromley, Kent, U.K.) equilibrated in the same buffer. The column was washed with 1.5 litres of the equilibrating buffer, after which C5 was eluted with 500 ml of 2.0 M-KCl/0.025 M-potassium phosphate/0.01 % NaN₃, pH 8.0. Next, 500 ml of starting buffer was passed through the column and the C3 eluted with a linear gradient formed with 1 litre of 0.1 M-KCl/0.025 M-potassium phosphate/0.01 % NaN₃, pH 8.0, and 1 litre of 0.1 M-KCl/0.20 M-potassium phosphate/0.01 % NaN₃, pH 8.0. The fractions containing C3 were pooled and were more than 90 % homogeneous as judged by electrophoresis on polyacrylamide gel. C1, C4, C2 and C5 could not be detected by haemolytic assay. C3 preparations were stored at 4°C in 0.1 % NaN₃ for months with little or no loss of haemolytic activity.

The haemolytic activities of whole-serum complement CH₅₀, C1, C2, C4, C3 and C5 were determined by the methods of Becker (1956), Borsos & Rapp (1967), Glovsky & Fudenberg (1970), Ruddy & Austen (1967) and Tack & Prahl (1976) respectively.

Polyacrylamide-gel electrophoresis

Electrophoresis of protein on polyacrylamide gel was performed by the method of Fairbanks *et al.* (1971), in sodium dodecyl sulphate-containing Tris/glycine buffers. Proteins were stained with Coomassie Brilliant Blue.

Antibody preparations

Rabbit anti-(sheep erythrocyte) serum was prepared as described by Mayer (1961). This antiserum was used directly to sensitize cells for haemolytic

complement component assays. An IgG fraction of this antiserum was also prepared as follows. The precipitate from 45% -satd. $(\text{NH}_4)_2\text{SO}_4$ was dissolved in phosphate-buffered saline and separated from IgM by chromatography on Sephadex G-200. No IgM was detected in the IgG-containing pools, as judged by double diffusion in agarose against a potent sheep anti-(rabbit IgM) serum.

An IgG fraction from horse anti-(rabbit IgG) serum was obtained by chromatography on Sephadex G-200 and an $(\text{Fab}')_2$ fraction containing only anti-(rabbit IgG) was purified by affinity chromatography and pepsin digestion for use in radioimmunoassays (Jensenius & Williams, 1974).

Antisera to human C1q, C1s, C4 and ovalbumin were prepared by immunization of rabbits with purified antigens. A first injection of 200 μg of antigen into the left thigh muscle was followed 1 week later by a second injection into the right thigh muscle. Finally, 100 μg of antigen was injected intravenously 7 days later and bleeding from the ear veins was started at weekly intervals thereafter. Against whole human serum in Ouchterlony double-diffusion assays (Ouchterlony, 1953) all antisera showed only one band and no cross-reaction with related proteins such as C1q, C1r, C1s, C4, C3, ovalbumin and rabbit IgG. Antibody $(\text{Fab}')_2$ fractions of the purified antibodies were also obtained as follows. Pure antigens were coupled to CL-Sepharose 6B after activation with CNBr (Cuatrecasas & Anfinsen, 1971). The antisera were passed through affinity columns containing their respective antigen. Specific antibodies were then eluted with 1M-propionic acid and adjusted to pH 7.8 with an equal volume of 1M-Tris/HCl. After dialysis against phosphate-buffered saline, $(\text{Fab}')_2$ fractions from antisera against components C1q, C1s and C4 were obtained by pepsin digestion (Fanger *et al.*, 1970). All $(\text{Fab}')_2$ antibody preparations showed inhibition only by their respective antigen in radioimmunoassays with human C1q, C1r, C1s, C4, C3, rabbit IgG and ovalbumin.

Antigen-antibody aggregates

Ovalbumin-anti-ovalbumin aggregates were formed with rabbit IgG anti-ovalbumin. The IgG fraction was obtained after three precipitations of antiserum with 45% -satd. $(\text{NH}_4)_2\text{SO}_4$, chromatography on DEAE-Sephadex A-50 in 0.03M-potassium phosphate, pH 8.0, and chromatography on Sephadex G-200 in phosphate-buffered saline. An $(\text{Fab}')_2$ fraction was obtained by pepsin digestion (Fanger *et al.*, 1970). Ovalbumin-anti-ovalbumin aggregates were formed with optimum amounts of ovalbumin at 4°C overnight in phosphate-buffered saline. The precipitates were washed three times in phosphate-buffered saline and finally resuspended in 0.15M-

NaCl/0.01% NaN_3 /1mM-di-isopropyl phosphorofluoridate. Immune aggregates were also prepared containing a mixture of IgG and $(\text{Fab}')_2$ anti-ovalbumin, and referred to as 'hybrid' aggregates. Hybrid aggregates containing 80% $(\text{Fab}')_2$ and 20% IgG (molar ratios) were prepared with optimal ovalbumin concentrations at 4°C overnight with constant stirring. Less than 5% of the original protein remained in solution, indicating that both types of antibodies were utilized in the aggregate formation.

Iodination of immunoglobulins

Radioiodination of antibody preparations with ^{125}I was done with chloramine-T (Byrt & Ada, 1969). Dithionite was omitted to avoid reduction of disulphide bonds in $(\text{Fab}')_2$ preparations. A 10 μl portion containing 40 μg of protein was added to 20 μl containing 1 mCi of carrier-free ^{125}I iodide and 20 μg of chloramine-T in 0.15M-potassium phosphate, pH 7.4. After 2 min at room temperature (16–20°C) 10 μg of tyrosine in 25 μl was added to stop protein iodination. Finally, carrier bovine serum albumin was added and free iodine separated on a column (10cm $\times$ 1cm) of Sephadex G-50. Antibodies were labelled to 20–30 $\mu\text{Ci}/\mu\text{g}$.

Radioimmunoassays

Radioimmunoassays to detect IgG, C1q, C1s and C4 were modified from the method of Morris & Williams (1975). The assay consisted of a test sample antigen inhibiting the binding of ^{125}I -labelled antibody to an antigen-coated 'target' cell. The target cells were glutaraldehyde-fixed erythrocytes having antigen bound to their surface (see below). Any labelled antibody not bound to the test sample antigen in a first incubation would bind to the antigen-coated target cells in a second incubation. These target cells were then washed and counted for radioactivity in a gamma counter. Specifically, dilutions of a test sample in 75 μl were incubated for 15–20 h at room temperature with 1.5×10^5 c.p.m. of ^{125}I -labelled $(\text{Fab}')_2$ antibody raised against the antigen to be detected. The samples were then centrifuged at 3000g for 30 min to pellet any labelled antibody bound to aggregates, and 100 μl of the supernatant was transferred to 50 μl containing 1×10^7 antigen-coated target cells. After 1 h at room temperature, the target cells were washed twice and finally counted for radioactivity in an LKB 1270 Rackgamma gamma counter. Low-ionic-strength buffer with 0.1% bovine serum albumin was used throughout.

The radioactivity of the target cells was plotted against the dilutions of the test sample. The 50% midpoint of the resultant sigmoidal binding curve was compared with a standard antigen curve to obtain the concentration of antigen in the sample. Samples

run in duplicates were always within 5% of each other.

Target cells

Rat or sheep erythrocytes were washed three times in balanced buffered salt solutions and resuspended to 2.5×10^8 cells/ml. Then 100 ml of cells and an equal volume of 0.25% glutaraldehyde were mixed for 5 min at room temperature; 20 ml of 10% (w/v) bovine serum albumin was then added, followed by immediate centrifugation at 1000g for 10 min and washing in balanced buffered salt solutions/0.1% bovine serum albumin. The fixed cells were finally suspended in saline to 2.5×10^8 cells/ml. Human C1q, C1s or C4 were linked to the fixed rat cells by the CrCl₃ method of Parish & Hayward (1974); 200 µg of the purified antigen was coupled per 25 ml of 2.5×10^8 cells/ml.

Rabbit IgG-target cells were prepared for use in the IgG radioimmunoassay by adsorbing an amount of rabbit anti-(sheep erythrocyte) IgG to fixed sheep cells, which would achieve a 70–80% binding of the total ¹²⁵I-labelled anti-IgG (Fab')₂ used in the radioimmunoassay. All target cells were stable for at least 2 months at -70°C.

Results

Comparison of complement activation by antibody bound to erythrocytes and in immune aggregates

Destruction of whole complement and C4 in serum

Titration of antibody binding to sheep erythrocytes showed that a maximum was reached at about 1.8×10^5 antibody molecules per cell, but at this concentration the cells were sticky and difficult to handle, so cells with 1×10^5 – 1.5×10^5 antibody molecules per cell were used routinely.

Increasing amounts of cell-bound or aggregated antibody were added to human serum, and both the total complement and the C4 remaining in the supernatant after incubation at 37°C for 1 h were assayed. Consumption of both whole complement and C4 followed a sigmoidal curve when similar amounts of either cell-bound or aggregated antibody were added (Fig. 1). The somewhat higher effectiveness of the cell-bound antibody may be due to its greater availability when spread on the cell surface compared with insoluble aggregates, but the small difference suggests that the cell surface as such has no direct role. These and subsequent haemolytic assays were accurate to $\pm 15\%$.

Ability to bind C1

The C1q and C1s bound to either cell-bound or aggregated antibody after incubation with partially purified C1 were measured by radioimmunoassay.

With increasing amounts of added C1, about 100 molecules of C1q and 200 molecules of C1s were bound per 1000 molecules of antibody in the aggregates, and again 40–100% higher amounts were bound to antibody on cells (Fig. 2). The ratio of C1s/C1q molecules bound in all experiments was in the range 1.5–2:1. Increasing the time of incubation or varying the Ca²⁺ concentration from 0.5 to 2.5 mM had no effect on the uptake. Lowering the NaCl concentration below 0.1 M increased C1 binding, but some non-specific precipitation occurred and the results were unreliable. Erythrocytes carrying 0.5×10^5 – 1.5×10^5 IgG molecules per cell showed less than 20% variation in C1 bound per µg of IgG bound.

The enzymic ability of C1 bound on either cells or aggregates was estimated by the ability of suspensions of either form to destroy haemolytic C4 in solution. It was found that activity per weight of bound C1 present was the same in both cases and in both was stable for over 16 h at 30°C.

Ability of cell-bound antibody and aggregated antibody together with C1 to bind C4

A marked difference in the ability to bind C4 was found between antibody-sensitized cells and antibody aggregates when both bound maximum amounts of

Fig. 1. Destruction of whole complement activity in serum (a) and component C4 (b) by addition of antibody-coated erythrocytes (○) and of ovalbumin-anti-ovalbumin aggregates (●).

Samples of either antibody-sensitized erythrocytes or antibody aggregates were incubated with 0.1 ml of human serum in a total volume of 0.5 ml for 60 min at 37°C. Residual (whole complement) CH₅₀ and C4 activity was determined by haemolytic titration on the supernatants after centrifugation. Δ, Equivalent number of cells without antibody.

Fig. 2. Binding of C1q (●) and C1s (○) to antibody-coated erythrocytes (a) and to ovalbumin-anti-ovalbumin aggregates (b)

Samples (2ml) of sensitized cells or antibody aggregates were incubated with increasing amounts of C1 in 0.1 M-NaCl/0.002 M-CaCl₂/0.01 M-Tris/HCl/2.5% glucose, pH 7.4, for 30 min at 30°C. After a washing, the amount of C1q or C1s bound was determined by specific radioimmunoassay. Controls of erythrocytes or heat-aggregated ovalbumin showed less than 2% and 8% uptake of C1s respectively compared with experimental samples. Erythrocytes carrying 0.5×10^5 – 1.5×10^5 IgG molecules per cell showed less than 20% variation in C1 bound per IgG molecule.

C1. If sensitized cells or aggregates were incubated with C4 for 30 min at 30°C and the bound C4 was estimated after a centrifuging and washing, both complexes showed increased binding with increasing C4 concentration (Fig. 3) and a maximum per 1000 molecules of antibody close to 1200 for aggregates and about 16000 for cell-bound antibody. This is in agreement with previous reports that activated C4 binds to the erythrocyte membrane (Müller-Eberhard & Lepow, 1965). If C1 was not present, negligible binding of C4 occurred in both cases. Furthermore, even when C1 was in excess, the binding of C4 was inefficient (only 2% for antibody in aggregates and 7% for cell-bound antibody),

Fig. 3. Binding of C4 to antibody-coated erythrocytes (a) and to ovalbumin-anti-ovalbumin aggregates (b) carrying near-maximal amounts of C1

Samples of either sensitized erythrocytes (320 C1s molecules bound/1000 IgG molecules) or antibody aggregates (120 C1s molecules bound/1000 IgG molecules) were incubated with increasing amounts of C4 for 30 min at 30°C. After washing of the cells and aggregates, bound C4 was determined by radioimmunoassay.

confirming earlier work by Müller-Eberhard *et al.* (1966) and Cooper & Müller-Eberhard (1968). This was in contrast with the binding of C1, which in antibody excess is more than 90% complete. The maximum uptake of C4 varied somewhat, depending on the preparation of C4 used, although the cells always bound 10–13-fold more C4 than did aggregates. This variability could not be traced to differences in purity, specific activity or age of the C4 preparations.

Effectiveness of C4 bound to sensitized cells and aggregates in the haemolytic system

There was a 10–13-fold difference between the relative amount of C4 bound between sensitized cells and aggregates carrying the same amount of antibody, but only a 1.5–2-fold difference in their ability to activate the whole complement system. This suggested

Fig. 4. Comparison of the binding of C4 with the development of C3 convertase activity on antibody-coated erythrocytes (a) and on ovalbumin-anti-ovalbumin aggregates (b).

Samples of either 10^7 sensitized cells carrying 1.3×10^{12} IgG and 4.3×10^{11} CIs molecules or aggregates containing 14×10^{12} IgG and 11×10^{11} CIs molecules were incubated with various amounts of C4 for 30 min at 30°C . Samples were removed to measure bound C4 by specific radioimmunoassay. After a washing and addition of 5000 units of C2 for 15 min at 30°C to develop the C3 convertase (C4₂), the samples were washed and incubated with 13 units of C3 for 30 min at 30°C . Finally, the cells or aggregates were separated by centrifugation and the residual C3 activity in the supernatant was determined by haemolytic assay. The percentage of C3 destroyed (●) and of C4 bound (○) is plotted versus the amount of C4 added to the samples.

that most of the C4 bound to sensitized cells might be ineffective in the complement cascade when interacting with C2 to form the C3 convertase enzyme and so continuing the sequence. Consequently, the C3 convertase activity formed by antibody-CI-C4 complex in the presence of excess C2 was measured with increasing concentrations of C4 and compared with the number of C4 molecules bound (Fig. 4).

With constant amounts of antibody and CI, the C3 convertase activity associated with either sensitized cells or aggregates reached a maximum much more quickly than did C4 binding. Furthermore, repeated experiments showed that peak convertase activity was obtained with only 7-10% of the maximum C4 bound to sensitized cells, and 50% bound to aggregates. The percentage of effective C4 molecules

forming C3 convertase was higher in aggregated than in cell-bound antibody. This suggested that only C4 bound to antibody, not to the cell surface, was able to form C3 convertase, though not all antibody-bound C4 is effective.

C4 activated by CI in solution has no haemolytic activity, and apparently rapid binding to antibody is essential for preservation of activity. Hence proximity of CI and antibody receptors for C4 may be an essential factor in both the binding and activity of C4. If so, the C4 bound to a constant amount of antibody would be proportional to the number of CI molecules present. This was found to be correct (Fig. 5). With both cell-bound antibody and with aggregates, radioimmunoassay showed that initially the C4 molecules bound increased linearly with increasing numbers of CIs molecules present, reaching a maximum at higher values [not apparent from the curve in Fig. 5(b), referring to binding by aggregate.]

When the C3 convertase activity in antibody aggregates was measured in a parallel experiment, this also was proportional to the number of CIs molecules present (Fig. 6), showing that the binding of both total and effective C4 molecules is proportional to the number of CI molecules present. This supports the view that rapidity of binding after activation is an essential feature in utilization of C4.

Attempt to locate the bound C4 molecule on antibody-antigen aggregates

It is known that C1 binds to the Fc portion of aggregated antibody (Ishizaka *et al.*, 1962), but where C4 binds is uncertain. Attempts to allocate the C4-binding site to the Fab or the Fc portion of the antibody were unsuccessful by hydrolysis of the complex with papain or other proteinases. Either little splitting occurred or C4 was destroyed. Therefore an indirect measure was made by using antibody-antigen aggregates made up of either whole antibody or whole antibody and (Fab')₂ fragments. The antibody and (Fab')₂ fragments were stirred during addition of antigen to ensure as intimate a mixture as possible during precipitation. Both whole antibody or mixed aggregates were then incubated with CI, washed, and finally C4 binding was measured after incubation with increasing C4 concentrations. Fig. 7 shows that, with constant bound CI, the C4 bound is the same in both whole antibody and in the mixed antibody-(Fab')₂ aggregates in the molar proportions 1:4. When (Fab')₂ aggregates were treated similarly, a low but significant binding of CI and of C4 occurred, possibly owing to a small contamination (< 5%) of the (Fab')₂ with whole antibody. The correlation of C4 binding with the Fab content, but not the Fc content, of the aggregates suggested that the C4 was attached

Fig. 5. Binding of C4 to antibody-coated erythrocytes (a) and to ovalbumin-anti-ovalbumin aggregates (b) carrying increasing amounts of C1, measured as C1s

Samples of either 6.5×10^7 antibody-sensitized erythrocytes or antibody aggregates ($7 \mu\text{g}$ of IgG) carrying increasing amounts of C1 were incubated with either 4.6×10^5 C4 molecules (cells) or 1.9×10^5 C4 molecules (aggregates) for 21 h at 30°C . After a washing, bound C4 was determined by specific radioimmunoassay.

Fig. 6. Development of C3 convertase activity by ovalbumin-anti-ovalbumin aggregates carrying increasing amounts of C1 (measured as C1s) but constant amounts of C4

Samples of antibody aggregates ($1.4 \mu\text{g}$ of IgG) carrying increasing amounts of C1 and constant amount of C4 (105 C4 molecules per 1000 IgG molecules) were incubated with 1×10^4 units of C2 for 15 min at 30°C to generate C3 convertase activity (C42). After a washing and incubation for 30 min at 30°C with 5.4 units of C3, residual C3 in the supernatant was haemolytically titred.

Fig. 7. Binding of C4 to ovalbumin-anti-ovalbumin aggregates, and to mixed antibody-(Fab')₂ ovalbumin-anti-ovalbumin aggregates

Increasing quantities of C4 were incubated at 30°C for 30 min with: aggregates formed with whole IgG antibody carrying 39 C1s molecules per 1000 IgG molecules (●); aggregates formed with 20% IgG and 80% (Fab')₂ fragments and carrying 38 C1s molecules per 1000 IgG molecules plus (Fab')₂ units (Δ); or aggregates formed with all (Fab')₂ fragments carrying 10 C1s molecules per 1000 (Fab')₂ fragments (□). After washing of the aggregates, bound C4 was determined by specific radioimmunoassay.

Fig. 8. Suggested assembly of C1, C4 and C2 molecules on an antibody-coated cell surface

Previous evidence (Porter, 1977) has shown that C1q binds to the C_H2 domain of aggregated IgG and that C1r₂-Ca²⁺-C1s₂ complex binds to the collagenous tails of C1q. Most C4 and C2 molecules activated by C1s will become inactivated (C4i and C2i) in solution, but some C4 will bind either to the antibody molecules (probably the Fab portion) or to the cell surface. Some C2 will bind to C4 on the Fab portion of the same IgG molecules as carry C1 or adjacent molecules. C4 molecules on the cell surface or on more distant IgG molecules will not bind C2, as it will be inactivated in solution before reaching them.

to the Fab portion of the antibody, but binding of C4 to C1 cannot be excluded by these results. However, the latter cannot be correct, as, when C4 binding to increasing amounts of aggregates is measured with C1 constant, it rises in proportion to the aggregates present and hence cannot be bound directly to C1.

When dithiothreitol was added to the mixed aggregates containing both (Fab')₂ and whole antibody, most of the material dissolved and the supernatant contained both Fab' and antibody as well as C4, as judged by electrophoresis on polyacrylamide gel in sodium dodecyl sulphate-containing buffers. This suggested that the aggregates were indeed mixed with both (Fab')₂ and whole antibody attached to the same antigen molecule, resulting in aggregate dissolution when the (Fab')₂ gave Fab' on reduction, causing disruption of the lattice, even though whole antibody molecules were also present. If separate aggregates had been formed between antigen with whole antibody or antigen with (Fab')₂, only the latter would be expected to go in solution.

Discussion

The evidence given here suggests that antibody, whether bound to erythrocytes or in aggregates with ovalbumin, has a similar efficiency in activating whole complement and specifically in binding enzymically active C1. Though only C1q and C1s have been measured, it is likely that similar results would be obtained with C1r. The 1.5-2-times higher values found for antibody bound to erythrocytes probably

reflects the greater availability of antibody molecules on the cell surface than in aggregates.

When C4 binding was estimated, the antibody-erythrocyte complex bound 10-13 times more molecules of C4 than did aggregates, indicating that much of the additional C4 can attach to the cell surface, as other evidence suggested (Müller-Eberhard & Lepow, 1965; Cooper & Müller-Eberhard, 1968; Müller-Eberhard & Biro, 1963). However, the amount of functionally active C4 when measured by the development of C3 convertase activity was maximal with only 7-10% of the total C4 molecules bound on the cells and 50% of these bound on the aggregates. Since a higher proportion of bound C4 is utilized on antibody aggregates compared with C4 on sensitized cells, it is probable that the effective C4 molecules in both cases are binding to antibody. However, per antibody molecule, C4 is more effective on sensitized cells than on aggregates, and may again only reflect the relative unavailability of antibody molecules in the aggregates. The percentage of C4 bound by antibody and C1 is small for both aggregates (2%) and cell-bound antibody (8%), in contrast with binding of C1 (more than 90%). The difference may be that C1 is much more stable in solution than C4, which after activation by bound C1 diffuses to be bound on a different part of the same antibody molecule, on other antibody molecules, or on the cell surface. Inactivation occurring during this diffusion time accounts for low C4-binding efficiency, and the efficiency might be expected to be lower for antibody aggregates than for cell-bound antibody, where the cell surface also offers a binding site.

The percentage of effective C4 molecules on antibody-antigen aggregates, measured in terms of development of C3 convertase activity, rises with amounts of C1 present with constant amounts of C4 molecules bound (Fig. 6). This suggests that the effective molecules are those in closest proximity to the C1, i.e. either on the same antibody molecule or on molecules closely adjacent (Opferkuch *et al.*, 1971). A likely reason for this is that rapid binding of C2 to the bound C4 is necessary for formation of complex C42 and, as C2 is also activated by bound C1, only closely adjacent C4 offers an effective site. Alternatively, the C42 complex, which can be formed in solution (Nagasawa & Stroud, 1977), is less stable than C4 and is destroyed if not bound rapidly to closely adjacent antibody molecules. If either postulate is correct, the number of bound C2 molecules would be expected to correspond closely to the number of effective C2 molecules, and this can be tested.

The site of binding of C4 and of the C42 complex is likely to be the Fab part of the antibody molecule. Chan & Cebra (1968) showed that, if antibody used to form an antigen-antibody precipitate was first 'pre-split' by resin-coupled papain and then the precipitate dissolved with reducing agent, the soluble Fab aggregates, but not Fc, gave a reaction with anti-C4 antiserum in double-diffusion gels. Subsequent elucidation of the disulphide-bond arrangement of antibodies makes the interpretation of their work uncertain. Our attempts to achieve mild conditions for the digestion of antibody aggregates without destruction of bound complement components were unsuccessful. However, the ability of aggregates, made with either whole antibody or a mixture of whole antibody and (Fab)₂ fragments, to bind similar amounts of C4 when carrying constant amounts of C1 suggests that C4 is in fact bound to the Fab part of the antibody molecule. This is in agreement with the conclusion of Chan & Cebra (1968), and, as other experiments showed that C4 binding increases with the increasing amounts of antibody aggregates present, when the C1 content was constant, the binding of C4 cannot be to C1 but is most likely to be to the Fab part of the molecule. A more direct demonstration of this point would, however, be desirable.

The diagram in Fig. 8 summarizes the suggested assembly of the early complement components. Earlier evidence summarized by Porter (1977) shows that the 'heads' of the C1q molecule bind to antibody via the C_H2 domain of Fc and that the C1r₂-Ca²⁺-C1s₂ tetrameric complex binds to the collagenous tails of C1q, leading to activation of C1s. C1s activates C4, a small fraction of which binds either to antibody or to the cell surface. Müller-Eberhard *et al.* (1967) have shown that, if C4 is bound to erythrocytes without antibody, subsequent addition of C2 and C1s in

solution followed by EDTA-treated serum to supply the late complement components will cause lysis. This indicates that cell-bound C4 can form complex C42 and continue the complement sequence if C2 is activated in solution, some of the active molecules being close to the bound C4.

The inefficiency of cell-bound C4 relative to antibody-bound C4 (Fig. 4) is therefore likely to be due to inactivation of C2 in diffusing the greater distance from the place of activation by C1s bound to C1q on the Fc portion of the antibody molecules to C4 molecules on the cell surface compared with C4 on the same or closely adjacent antibody molecules. If this interpretation of the results is correct, the cell-bound C4 molecules will be capable of forming C3 convertase, but in practice the C2 molecules will be inactivated before reaching them. Alternatively, if C42 diffuses as a complex C2 will be inactivated before C4 binds to the cell.

We thank Dr. I. Gigli for her most valuable help with several of the experiments reported in this paper and Dr. K. B. M. Reid for his advice and gifts of reagents. J. W. F. G. held a U.S. National Institutes of Health Postdoctoral Fellowship.

References

- Becker, E. L. (1956) *J. Immunol.* **77**, 462-468
- Borsos, T. & Rapp, H. J. (1967) *J. Immunol.* **99**, 263-268
- Borsos, T., Rapp, H. J. & Mayer, M. M. (1961) *J. Immunol.* **87**, 310-325
- Byrt, P. & Ada, G. L. (1969) *Immunology* **17**, 503-516
- Chan, P. C. Y. & Cebra, J. J. (1968) *Immunochemistry* **5**, 1-8
- Cooper, N. R. & Müller-Eberhard, H. J. (1968) *Immunochemistry* **5**, 155-169
- Cooper, N. R. & Müller-Eberhard, H. J. (1970) *J. Exp. Med.* **132**, 775-793
- Cuatrecasas, P. & Anfinsen, C. B. (1971) *Annu. Rev. Biochem.* **40**, 259-278
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606-2617
- Fanger, M. W., Hart, D. A., Wells, J. V. & Nisonoff, A. (1970) *J. Immunol.* **105**, 1484-1492
- Fothergill, J. E. & Anderson, W. H. K. (1978) *Curr. Top. Cell. Regul.* in the press
- Gigli, I., Porter, R. R. & Sim, R. B. (1976) *Biochem. J.* **157**, 541-548
- Gigli, I., von Zabern, I. & Porter, R. R. (1977) *Biochem. J.* **165**, 439-446
- Glovsky, M. & Fudenberg, H. H. (1970) *J. Immunol.* **104**, 1072-1078
- Goldlust, M. B., Shin, H. S., Hammer, C. H. & Mayer, M. M. (1974) *J. Immunol.* **113**, 998-1007
- Ishizaka, K., Ishizaka, T. & Sugakara, T. (1962) *J. Immunol.* **88**, 690-701
- Jensenius, J. C. & Williams, A. F. (1974) *Eur. J. Immunol.* **4**, 91-97
- Kerr, M. & Porter, R. R. (1978) *Biochem. J.* **171**, 99-107
- Mayer, M. M. (1961) in *Experimental Immunochemistry* (Kabat, E. A. & Mayer, M. M., eds.), 2nd edn., pp. 133-240, Charles C. Thomas, Springfield

- Morris, R. J. & Williams, A. F. (1975) *Eur. J. Immunol.* **5**, 274–281
- Müller-Eberhard, H. J. (1975) *Annu. Rev. Biochem.* **44**, 697–724
- Müller-Eberhard, H. J. & Biro, C. E. (1963) *J. Exp. Med.* **118**, 447
- Müller-Eberhard, H. J. & Lepow, I. H. (1965) *J. Exp. Med.* **121**, 819–833
- Müller-Eberhard, H. J., Dalmasso, A. P. & Calcott, M. A. (1966) *J. Exp. Med.* **123**, 33–54
- Müller-Eberhard, H. J., Polley, M. J. & Calcott, M. A. (1967) *J. Exp. Med.* **125**, 359–380
- Nagasawa, S. & Stroud, R. M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2998–3001
- Nelson, R. A., Jenson, J., Gigli, I. & Tamura, N. (1966) *Immunochemistry* **3**, 111–135
- Nilsson, U. R. & Müller-Eberhard, H. J. (1965) *J. Exp. Med.* **122**, 277–298
- Opferkuch, W., Rapp, H. J., Colten, H. R. & Borsos, T. (1971) *J. Immunol.* **106**, 407–413
- Ouchterlony, Ö. (1953) *Acta Pathol. Microbiol. Scand.* **32**, 231
- Parish, C. R. & Hayward, J. A. (1974) *Proc. R. Soc. London Ser. B* **187**, 47–63
- Polley, M. J. & Müller-Eberhard, H. J. (1968) *J. Exp. Med.* **128**, 533–551
- Porter, R. R. (1977) *Biochem. Soc. Trans.* **5**, 1659–1673
- Porter, R. R. (1978) in *Defence and Recognition* (Lennox, E. S., ed.), Butterworths, London, in the press
- Reid, K. B. M. (1974) *Biochem. J.* **141**, 189–203
- Ruddy, S. & Austen, K. F. (1967) *J. Immunol.* **99**, 1162–1172
- Shortman, K. (1968) *Aust. J. Biol. Med. Sci.* **46**, 375–396
- Tack, B. F. & Prahl, J. W. (1976) *Biochemistry* **15**, 4513–4521
- World Health Organisation (1968) *Bull. W.H.O.* **39**, 935–938