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Effector Activating Determinants on IgG

II. DIFFERENTIATION OF THE COMBINING SITES FOR C1q FROM THOSE FOR CYTOTOXIC K CELLS† AND NEUTROPHILS BY PLASMIN DIGESTION OF RABBIT IgG

I. C. M. MacLennan, G.E. Connell* and Frances M. Gotch

Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford

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Summary. Plasmin digestion of rabbit IgG after pretreatment at low pH yields a fragment (Facb) which has lost the C_H3 domain. A study of the biological activity of this fragment is described and the following conclusions are drawn: (1) Facb possesses the same antigen-combining capacity as the whole antibody; (2) C1 fixation is normal; (3) The capacity to sensitize target cells to lysis by cytotoxic K cells is lost; (4) Facb–antigen complexes are unable competitively to inhibit phagocytosis of sensitized bacteria by neutrophils.

INTRODUCTION

Many immunological effector mechanisms such as the complement system, phagocytosis by neutrophils and macrophages, degranulation of basophils, and target cell killing by cytotoxic K cells can be specifically activated by antigen through IgG. However, not all mechanisms are activated by the same conditions of antigen–antibody combination. For instance, sensitization of target cells with specific IgG will often render cells vulnerable to lysis by cytotoxic K cells without making them susceptible to complement-mediated lysis (MacLennan, Loewi and Howard, 1969). None of these mechanisms are activated, however, by native IgG and some physical alteration which may result from either aggregation, conformational change of IgG molecules or a combination of both of these processes is required for activation. The present studies have been concerned with the conditions required for activation of immunological effector mechanisms. The first paper (MacLennan, Howard, Gotch and Quie, 1973) provided evidence as to which of the subclasses of human IgG activated various effector mechanisms. This second communication describes some findings in relation to the location of effector activating sites on IgG molecules. Pepsin digestion of IgG yielding the $F(ab')_2$ fragment is associated with loss of C1 activation (Reid, 1971), neutrophil activation (Quie, Messner and Williams, 1968) and cytotoxic K cell activation (Möller and Svehag, 1972) while the alternative pathway of complement fixation through C3 remains intact (Reid, 1971). Plasmin digestion of rabbit IgG following pretreatment of the molecule at low pH results in cleavage of the γ chains of the molecule at a site nearer the C terminal than that achieved with

* Faculty of Medicine, University of Toronto, formerly Visiting Research Fellow of Department of Biochemistry, South Parks Road, Oxford.

Correspondence: Dr I. C. M. MacLennan, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford.

† Formerly termed cytotoxic 'B' cells.

pepsin (Connell and Porter, 1971). We have tested the biological activity of a fragment (Facb) derived from IgG by plasmin digestion and we show that the C1 activating determinant remains intact following this procedure while the cytotoxic K cell and neutrophil activating determinants are lost.

MATERIALS AND METHODS

Antisera

Rabbit anti-Chang serum was prepared by the intradermal injection of 10^7 washed Chang cells in Freund's complete adjuvant over the left shoulder followed by intravenous injections of 10^7 Chang cells at 3 and 5 weeks after the primary injection. The serum was harvested 2 weeks after the last injection.

Rabbit anti-human serum albumin (HSA) was prepared by injection of 10 mg HSA in Freund's complete adjuvant subcutaneously and in the foot pads of each of six rabbits. Three months later each rabbit received 5 mg HSA in Freund's incomplete adjuvant and the animals were bled 10 days later.

Anti-*Streptococcus viridans* was obtained from a patient (M.T.) with a subacute bacterial endocarditis.

Antigens

HSA was obtained from Koch Light purified batch (0145t). ^{125}I-labelled HSA was prepared by the iodine monochloride technique. Chang cells were cultured in suspension and where appropriate they were labelled with ^{51}Cr as described previously (MacLennan and Loewi, 1968).

Preparation of rabbit IgG

IgG was precipitated from rabbit serum by adding saturated aqueous ammonium sulphate in the cold to give a final concentration of 0·375 saturation. The precipitate was collected and redissolved in 4/10 of the original volume of 0·20 M Tris–HCl buffer, pH 8·2. The IgG was reprecipitated at the same concentration of ammonium sulphate and the same procedure was repeated for a third precipitation. The final precipitate was redissolved in a minimum of 0·03 M sodium phosphate buffer, pH 7·40, and dialysed against several changes of that buffer. The solution was then added to DEAE–cellulose (Whatman DE-32) which had been equilibrated against the same buffer. The non-absorbed fraction was adjusted to a concentration of 20 mg protein per ml assuming an extinction coefficient at 1 mg/ml (280 mμ/1 cm), of 1·37.

IgG was prepared separately from anti-Chang serum and from anti-HSA serum. The two preparations were then pooled in a ratio (w/w) of 2 : 1 anti-HSA : anti-Chang. The final pool had inherent plasmin activity of 7·0 units per mg. IgG as assayed with benzolarginine ethyl ester by the method of Schwert and Takenaka (1955).

Plasmin digestion

IgG at 20 mg/ml was taken to pH 2·5 by the addition of 1 M HCl and incubated at 30° for 60 minutes. At the end of this period the solution was restored to pH 7·0 by the addition of 1 M NaOH and incubated for a further 10 minutes at 30°. Digestion was stopped by the addition of 400 mg urea per ml of reaction mixture. Facb was separated from residual IgG and from the other major digestion product by recycling gel filtration

on Sephadex-G150 as described by Connell and Porter (1971). The Facb fraction was dialysed in the cold against several changes of water, and then against 0·10 M NaCl–0·05 M Tris–HCl, pH 7·5.

Under the digestion conditions employed the inherent 'plasmin' activity converts not more than 25 per cent of the IgG to Facb as judged by starch gel electrophoresis in acid–urea buffer (Smithies, Connell and Dixon, 1962). IgG treated at low pH and high urea concentrations but omitting incubation at pH 7·0 was used as a further control in the experiments cited in the results. These data are not included as this procedure gave a product functionally identical to the original IgG.

Assay of the titre of anti-Chang antibody capable of sensitizing Chang cells to lysis in the presence of cytotoxic K cells

Antisera were diluted in Minimum Essential Medium with 10 per cent foetal bovine serum (MEM10) in 0·5 ml volumes, ½ ml of a suspension in MEM10 containing 10^4 ^{51}Cr-labelled Chang cells and 2×10^5 human peripheral blood lymphocytes was added to the tubes containing antibody. The cultures were incubated for 4 hours and then centrifuged at 400 *g* for 10 minutes at 4° after which half the supernatant was removed. By counting the ^{51}Cr activity in this aliquot and the residue of the culture the percentage of ^{51}Cr release was determined. The marked prozone seen with rabbit anti-Chang antibody is largely attributable to the activity of this antibody against human cytotoxic K cells. This effect, however, is titred out well before that of the cytotoxicity inducing antibody.

Complement fixation test

This was performed using guinea-pig complement (Wellcome freeze-dried), formalized sheep red blood cells (Wellcome) and rabbit anti-sheep red blood cell haemolytic antibody (Wellcome). HSA : anti-HSA mixtures were diluted in complement-fixing buffer, pH 7·2 (Oxoid). The optimal ratio of anti-HSA to HSA for complement fixation with these preparations was found to be 10 : 1. This ratio was used in all experiments. The degree of dissociation of complexes over the dilution range used was not sufficient to warrant alteration in ratio or dilution. The dilutions in 0·1 ml were added to precipitin tubes containing 0·1 ml of a solution of complement which was of sufficient strength just to lyse the sheep red cells in 0·1 ml of a 0·4 per cent solution of sensitized sheep red cells. The antigen, antibody and complement were incubated for 16 hours at 4°. At the end of this time the indicator red cells which had been ^{51}Cr-labelled were added and the mixture was incubated for a further 2 hours at 37°. The tubes were then centrifuged, an aliquot removed and the percentage of ^{51}Cr release determined as described above. The results were expressed as the titre which was able to produce 50 per cent inhibition of sheep red cell lysis.

Assay of anti-HSA antibody-combining activity

This was carried out using the ammonium sulphate precipitation method of Farr (1958). Anti-HSA IgG or Facb was diluted in borate buffer, pH 8·5 and 0·1 ml aliquots of the dilutions were added to 1 μg of ^{125}I-labelled HSA in 0·1 ml of the same buffer enriched with 10 per cent normal rabbit serum. The mixture was incubated at 4° for 18 hours at which time 0·2 ml of saturated ammonium sulphate was added to and thoroughly mixed with the contents of each tube. One hour later the tubes were centrifuged at

1500*g* at 4° for 30 minutes. Half the supernatant was removed and the percentage of counts precipitated was determined as in the other radio-immunoassays described above.

Assays of intra-cellular killing of bacteria sensitized with IgG in the absence of complement

The bacteria used in this assay were *Streptococcus viridans* from patient M.T. Five million of these organisms in 0·1 ml of Herpes buffered basal medium enriched in 0·1 per cent gelatine (HBg) were incubated with either 0·4 ml of a 1:50 dilution of heat-inactivated M.T. serum in HBg or 0·4 ml HBg for 30 minutes at 37°. After this they were centrifuged at 200 *g* for 5 minutes and the supernatant fluid removed. The bacteria were then suspended in 0·5 ml HBg (which where appropriate contained inhibitory additives) and 0·5 ml of a suspension of 5×10^6 human peripheral blood polymorphs in HBg. These mixtures were then incubated at 37° and continuously tumbled on a rotary mixer. At 0 and 20 minutes 10 μl was removed from each tube and this was diluted in 2 ml distilled water which disrupted the polymorphs. One tenth of a ml of this diluted suspension was mixed with 1 ml fresh defibrinated sheep red cells and was plated out with 10 ml sterile columbia agar in a Petri dish. These cultures were incubated for 24 hours at 37° when the number of colonies per plate was then counted. The results are expressed as the count in cultures from tubes taken at 20 minutes as a percentage of the counts in time 0 cultures.

RESULTS

ESTIMATION OF ANTIBODY BINDING CAPACITY OF WHOLE AND PLASMIN DIGESTED RABBIT IGG

For the present study rabbit anti-HSA IgG was mixed with rabbit anti-Chang IgG in a 2 : 1 ratio. The purpose of mixing the antibodies was to ensure equivalent digestion of antibody used in the cytotoxic K cell assay and antibody used for complement fixation and neutrophil inhibition tests. The HSA-binding capacity of IgG compared with Facb preparations was compared using the Farr test. The results of such a test are shown in Fig. 1. It will be seen that equal optical density solutions ($E^{280}_{1\,cm}$) of the digest (Facb) and whole IgG preparations have almost the same antigen-binding capacities. All subsequent

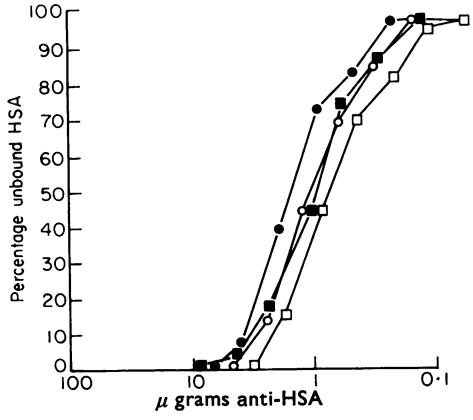

FIG. 1. Antibody-combining activity of whole IgG and Facb preparations as assessed by per cent binding of ^{125}I-labelled HSA. (●—●) Anti-HSA IgG. (○—○) Anti-HSA/Chang IgG. (■—■) Anti-HSA Facb. (□—□) Anti-HSA/Chang Facb. Each tube contained 1 μg ^{125}I-labelled HSA. Bound antigen was precipitated with half saturated ammonium sulphate.

tests are carried out with preparations adjusted to equal optical density ($E_{1\,cm}^{280}$) as this represented equivalent antigen-binding capacities as assessed by the Farr test.

THE COMPARATIVE CAPACITY OF Facb AND WHOLE IgG TO INDUCE ANTIBODY-DEPENDENT KILLING OF CHANG CELLS BY CYTOTOXIC K CELLS

Anti-Chang/HSA whole IgG and Facb preparations were assayed for their capacities to induce killing of Chang cells by cytotoxic K cells. The results are shown in Fig. 2. It

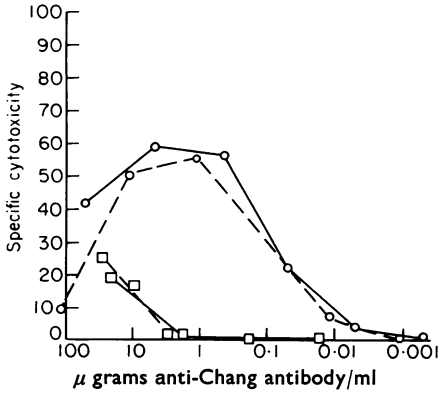

FIG. 2. The effect of plasmin digestion on the capacity of anti-Chang IgG to induce cytotoxic K cell killing of Chang cells. (○—○) Anti-HSA/Chang IgG. (□—□) Anti-HSA/Chang Facb. (○- -○) Anti-Chang IgG. (□- - -□) Anti-Chang Facb.

will be seen that in two separate digests a 600-fold drop in activity occurred. Further experiments were carried out to ensure that the antibody-combining activity of anti-Chang Facb was retained. These experiments showed that anti-Chang Facb could successfully compete with whole anti-Chang IgG for antigenic sites on the Chang cells. One in 10^4 anti-Chang antibody was mixed with dilutions of anti-Chang Facb. The sensitizing capacity of the anti-Chang antibody was diminished by anti-Chang Facb but not anti-HSA Facb (Fig. 3).

THE CAPACITY OF Facb ANTIBODY FRAGMENTS TO REACT WITH RECEPTORS FOR IgG ON NEUTROPHILS

Immune complexes made with IgG antibody reduce the rate of phagocytosis of sensitized bacteria by neutrophils and consequently the rate of intracellular killing of the bacteria is slowed. This results from competition for receptors for IgG on the neutrophils with IgG complexed to bacterial antigens. This effect can also be produced with aggregated IgG but not native monomeric IgG (MacLennan *et al.*, 1973). Experiments were set up to assess the capacity of HSA–anti-HSA complexes to inhibit phagocytosis of sensitized bacteria. Complexes were made with both monomeric anti-HSA/Chang IgG + HSA and anti-HSA/Chang Facb + HSA. The optimal inhibitory ratio of HSA–anti-HSA/Chang was used. This was determined by serial dilutions of HSA against a fixed concentration of anti-HSA/Chang IgG.

Anti-HSA, whole IgG and Facb alone had no inhibitory capacity in their own right.

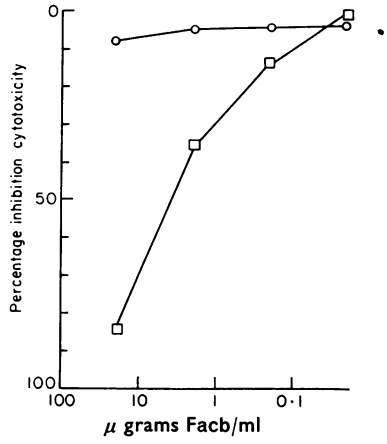

FIG. 3. The antigen-combining capacity of anti-Chang Facb. Chang cells were sensitized with 1: 10,000 anti-Chang antibody. (□—□) Anti-Chang Facb but not (○—○) anti-HSA Facb inhibited killing by cytotoxic K cells. This effect is consistent with anti-Chang IgG competing for antigenic sites on the Chang cell.

Complexes of HSA–anti-HSA/Chang IgG inhibited the killing of bacteria while equivalent amounts of HSA–anti-HSA/Chang Facb showed no inhibitory activity. The inability of Facb complexes made up with 100 μg of antibody per culture to show any inhibition precluded precise establishment of the loss of neutrophil-combining activity associated in the plasmin digestion. However, Facb anti-HSA/HSA complex failed to show any inhibition of phagocytosis at ten times the concentration of whole anti-HSA/HSA required to produce 50 per cent inhibition at 20 minutes. These results were reproduced with four experiments.

THE CAPACITY OF HSA–ANTI-HSA/CHANG Facb TO FIX C1

The conditions of these complement fixation tests were not compatible with C3 activation through the alternative pathway. The reasons for this were (a) that the tests were carried out at 4° (Reid, 1971) and (b) that the concentration of complement was inadequate to activate the alternative pathway.

The optimal ratio of HSA to anti-HSA/Chang for complement fixation was determined by serial dilution of HSA against a fixed concentration of anti-HSA/Chang whole IgG. The complement-fixing capacity of complexes made with whole and digested anti-HSA/Chang are shown in Fig. 4. In this experiment monomeric whole anti-HSA/Chang had not been separated from aggregates. It will be seen that the whole IgG in the absence of HSA has some complement-fixing activity. The small difference in the complement fixation titre shown with whole anti-HSA/Chang complexes compared to Facb anti-HSA/Chang complexes is entirely attributable to this effect. Both whole and Facb antibody preparations were subsequently centrifuged at 140,000 *g* (mean) for 7 hours at a concentration of $E_{1\,cm}^{280}$ of 1·0 in complement-fixing buffer. The complement-fixing capacities of these centrifuged preparations when complexed with HSA were then the same.

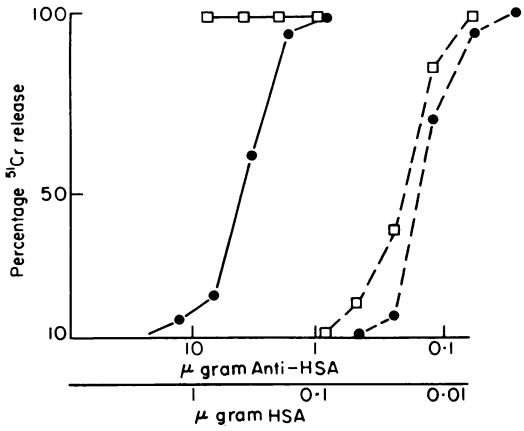

FIG. 4. The complement-fixing capacity of plasmin-digested IgG. The degree of inhibition of complement-mediated, sensitized red cell lysis caused by HSA anti-HSA complexes is shown. Lysis of sensitized red cells is assessed by ^{51}Cr release. Complexes are made up at the optimum ratio for complement fixation. (□—□) Facb anti-HSA/Chang. (●—●) Whole anti-HSA/Chang. (□- -□) Facb anti-HSA/Chang+HSA. (●- -●) Whole anti-HSA/Chang+HSA. The difference between the Facb and whole IgG preparations is entirely attributable to aggregation of whole IgG (see text). Monomeric preparations show identical complement-fixing activity.

DISCUSSION

The data presented in the results indicate that the determinants of rabbit IgG that activate neutrophils to phagocytose sensitized bacteria and induce cytotoxic K cells to lyse sensitized target cells differ from the C1q-binding sites. Plasmin digestion following treatment at pH 2·5 for 60 minutes destroys areas of the IgG molecule vital for the activation of neutrophils and Chang cells. Analysis of the anatomical nature of the plasmin disruption suggests that the major effect is to cleave the γ chains between the C_H2 and C_H3 domains of the heavy chains (Connell and Porter, 1971). One may conclude either that the lost activating sites are situated entirely within the C_H3 domains or that elements of that domain make a vital contribution to the activating sites.

When the complement fixation results are compared with that of other analyses it seems likely that the C1q-binding site can be localized to an area of not larger than eighty amino acid residues on the γ chain. Taranta and Franklin (1961) and Ishizaka, Ishizaka and Sugahara (1962) showed that Fc fragment obtained by standard papain digestion (Porter, 1959) possesses complement-fixing sites. Utsumi (1969) produced a shortened Fc fragment (m Fc) by prolonged digestion at neutral pH of rabbit IgG whose labile inter-heavy-chain disulphide bridges had been reduced. This fragment, which did not contain the hinge region, retained the complement binding activity at 4° of whole Fc but lost guinea-pig skin-binding activity. The C1q-binding site on positive evidence, therefore, would appear to reside entirely between the mFc cleavage point and the plasmin cleavage point.

It is possible that IgG molecules that carry neutrophil and cytotoxic K cell combining sites are of a class that is resistant to plasmin digestion, and that Facb derived from such a class does not appear in the product recovered under the present conditions. It is clear, however, at least with reference to human IgG, that both subclasses which bind C1q (IgG1 and IgG3) also activate cytotoxic K cells and neutrophils. In addition neutrophils are

activated by IgG2 and cytotoxic K cells by IgG2 and IgG4 (MacLennan *et al.*, 1973). If the human data are applicable to the rabbit then it would seem unlikely that there are IgG molecules with the capacity to bind C1q which have no capacity to activate neutrophils or cytotoxic K cells.

Although no data are presented in this paper on macrophage activation, definitive data have been provided by Yasmeen, Ellerson, Dorrington and Painter (1973) showing that the C_H3 domain of human IgG inhibited rosette formation on macrophages by IgG-coated red cells. When C_H3 itself was bound to red cells it formed rosettes with guinea-pig peritoneal macrophages. C_H2 fragments were negative in both these tests. Stewart, Smith and Stanworth (1973) have also investigated the biological activity of rabbit Facb. They report that the fragment fails to bind rheumatoid factor or fix to guinea-pig skin. Interaction of C_H3 of human origin with guinea-pig mast cells was noted by Minta and Painter (1972). These data suggest that the C_H3 domain of rabbit IgG is important in activating at least five immunological effector mechanisms. This represents a marked change from the conclusion drawn by Prahl (1967) that the C-terminal region of the Fc fragment does not carry biological activity other than that of species-specific determinants.

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