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Investigation of the Binding Site of Mouse IgG Subclasses to Homologous Peritoneal Macrophages

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Summary. The binding of mouse myeloma IgG1, IgG2a, IgG2b, IgG1 Fc, IgG2b Fc and a pepsin produced C-terminal subfragment of IgG1 Fc and IgG2b Fc (provisionally identified as pFc′) to mouse peritoneal macrophages was investigated. The high affinity cytophilic antibodies belonged to IgG2 subclasses and the binding site of these antibodies was located in the C_H3 homology region.

INTRODUCTION

Some immunoglobulins are cytophilic to macrophages (Boyden and Sorkin, 1960, 1961; Boyden, 1964) and the biological and physicochemical properties of these have been investigated (Berken and Benacerraf, 1966). In man IgG1 and IgG3 are cytophilic to monocytes (Huber and Fudenberg, 1968; Huber, Douglas, Nusbacher, Kochwa and Rosenfield, 1971; Hay, Torrigiani and Roitt, 1972) while guinea-pig and mouse cytophilic antibodies were found in the 7S γ2 fraction (Berken and Benacerraf, 1966; Nelson and Mildenhall, 1968; Parish, 1965). The subclass specificity of mouse immunoglobulins to peritoneal macrophages has been investigated (Hay, 1972) and it has been shown that IgG2a and IgG2b are cytophilic.

Cytophilic binding is a property of the Fc region of immunoglobulins. The $F(ab')_2$ fragments were not cytophilic, demonstrating the importance of Fc (Berken and Benacerraf, 1966; Inchley, Grey and Uhr, 1970). In guinea-pigs the cytophilic activity was directly shown to be present in the Fc portion of the IgG molecule (Liew, 1971).

Attempts to characterize the submolecular site involved in cytophilic binding has resulted in conflicting results. In the human system, this property seems to be a function of the C_H3 domain (Yasmeen, Ellerson, Dorrington and Painter, 1973; Okafor, Turner and Hay, 1974). In contrast to the above groups, Holm (1974) has reported that the C_H3 fragment is not capable of inhibiting monocyte-mediated haemolysis. Furthermore, studies with guinea-pig cytophilic IgG2, have suggested that binding is not mediated via the C_H3 region (Leslie, 1974). The object of the present work was to determine the binding site of mouse cytophilic antibodies to homologous peritoneal macrophages.

MATERIALS AND METHODS

Myeloma proteins

Mouse myeloma proteins were prepared by a modification of the method of Torrigiani

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(1972). Sera from mice bearing the tumours RPC 23 (IgG1), 5563 (IgG2a) and MOPC 141 (IgG2b) were purified by ion-exchange chromatography on DEAE–cellulose (phosphate buffer, pH 8·1) and gel filtration on Sephadex G-200 at pH 4·8 (acetate buffer, 0·1 M).

Enzymatic fragments

Fc of IgG1 and IgG2b were prepared by papain digestion of myeloma IgG1 and IgG2b as described by Gorini, Medgyesi and Doria (1969). The pFc′ fragments of IgG1 and IgG2b (corresponding to the C_H3 homology regions) were prepared by pepsin digestion (Dissanayake and Hay, 1975). Fab was obtained by papain digestion of normal mouse immunoglobulin.

Experimental animals

Peritoneal macrophages and normal mouse serum immunoglobulin (NMIg) were obtained from outbred CDI mice. Mouse anti-sheep red cell antiserum was raised in BALB/c mice.

Medium (MEM-FCS)

Eagle's minimum essential medium (MEM) (Wellcome Reagents, Beckenham, Kent) was used with the addition of heparin (5 i.u./ml) and 10 per cent heat-inactivated foetal calf serum (FCS) (Tissue Culture Services, Slough, Bucks.).

Macrophages

CDI mice were injected i.p. with 5 ml of a 5 per cent starch suspension. After 3 days, the peritoneal cavity was washed with 10 ml of MEM–FCS medium. The cells were spun at 200 ***g*** for 10 minutes, washed twice with MEM–FCS medium and the concentration adjusted to 10^6 cells per millilitre.

Mouse anti-sheep red cell serum

BALB/c mice were primed with 2×10^8 sheep red blood cells (Wellcome Reagents, Beckenham, Kent) in Freund's complete adjuvant (FCA) given by intraperitoneal injection. At 10-day intervals, two boosting injections of 2×10^8 cells in FCA were given. Ten days after the last injection, the mice were exsanguinated under ether anaesthesia.

Radiolabelling of myeloma proteins

Myeloma proteins and enzymatic fragments in phosphate-buffered saline (PBS) (1 mg/ml), were incubated with ^{131}iodine ($Na^{131}I$) (IBS 30, Radiochemical Centre, Amersham, Bucks.) (0·1 mCi/mg protein) with 10 μl of chloramine T solution (6 mg/ml) for 2 minutes at room temperature. The reaction was stopped by adding 10 μl of sodium metabisulphite solution (12 mg/ml). The iodinated protein was separated from free iodine by gel filtration on a Sephadex G-25 column and dialysed exhaustively against PBS at 4°. Prior to labelling, all the protein preparations were run through a calibrated Sephadex G-150 column to remove aggregated material. Radiolabelling of this aggregate free material did not cause any detectable aggregation as checked by gel filtration on Sephadex G-150.

Direct binding of labelled immunoglobulins to peritoneal macrophages

One-millilitre aliquots of macrophage suspension (10^6 cells per millilitre) in MEM–FCS

were incubated with 0·3 g of washed glass beads (BDH Glass Beads for gas chromatography, approximately 60 mesh), in plastic tubes for 1 hour at 37° in 5 per cent CO_2 in air. Non-adherent cells were washed away with two changes of MEM–FCS medium and cooled to 4°. Varying amounts of labelled protein were added to duplicate tubes containing glass beads with macrophages and incubated at 4° for 1 hour in 5 per cent CO_2 in air. The tube contents were then washed with five changes of PBS and counted for radioactivity in a Packard Autogamma Counter. Corrections were made for the non-specific sticking of labelled proteins to glass beads and plastic tubes by incubating equal amounts of labelled proteins with glass beads without macrophages. Counts obtained with these were subtracted from those containing macrophages. The results are expressed as the number of micromoles of labelled protein bound to 10^6 macrophages.

Inhibition of binding of labelled immunoglobulins with Fc and pFc' of IgG1 and IgG2b

Macrophages were stuck to glass beads as described and incubated with 300×10^{-7} μmoles of labelled IgG1 and IgG2b and the binding was inhibited by simultaneous addition of varying amounts of unlabelled IgG1, IgG1 Fc, IgG1 pFc', IgG2b, IgG2b Fc and IgG2b pFc'. Incubations, washings and correction for non-specific binding were the same as in the direct binding technique. The extent of inhibition was expressed as the percentage bound relative to that in the absence of inhibitory protein.

Macrophage cytophilic rosettes

0·5 ml of mouse peritoneal exudate cell suspension in MEM–FCS medium (10^6 cells per millilitre) were placed in circular chambers composed of glass rings (14 mm diameter, 5 mm height) stuck to glass cover slips with 'Araldite'. The chambers were then kept in air-tight plastic boxes gassed with 5 per cent CO_2 in air and incubated at 37° for 1 hour. The non-adherent cells were freed from the macrophages stuck to the glass coverslips by washing with two changes of MEM–FCS medium. 0·5 ml of mouse anti-sheep red cell serum, diluted 1/40 with MEM–FCS medium was added to each chamber and incubated at 37° for 1 hour and at 4° for a further 30 minutes. The free antiserum was then washed away with two changes of medium and 0·5 ml of 0·5 per cent sheep red cell suspension was added to each chamber. The chambers were incubated at 4° for 1 hour in 5 per cent CO_2 in air. The free red cells were removed by washing carefully and the number of rosettes counted. Macrophages binding more than five red cells were taken as positive.

Inhibition of cytophilic rosettes

To determine the cytophilic rosette inhibiting capacity of the subclass proteins and enzymatic fragments, anti-SRBC serum was mixed with various amounts of subclass immunoglobulins and enzymatic fragments and added to the macrophages as described. The percentage inhibition was calculated as: percentage inhibition = [(1 − number of rosettes obtained per 100 macrophages)/(number of rosettes in the control per 100 macrophages)] × 100.

RESULTS

DIRECT BINDING

The results of direct binding of labelled IgG1, IgG2a and IgG2b are shown in Fig. 1. The binding was studied over a range of added protein concentrations varying from $4{\cdot}4 \times 10^{-7}$ to $5{\cdot}6 \times 10^{-5}$ μmoles per 10^6 macrophages. In all direct binding experiments it

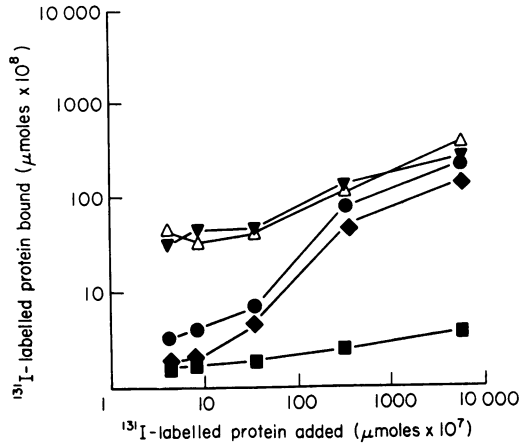

FIG. 1. Binding of ^{131}I-labelled mouse myeloma immunoglobulins to 10^6 mouse peritoneal macrophages. (▼) IgG2a. (△) IgG2b. (●) IgG1. (◆) NMIg (normal mouse immunoglobulin isolated on DE-52 cellulose). (■) Fab (papain-produced Fab from NMIg).

was found that the amount of labelled immunoglobulin bound to macrophages was dependent on the added protein concentration and significant differences in the amounts bound in the different subclasses were obtained at concentrations lower than 300–500×10^{-7} μmoles per 10^6 cells. Similar observations have been reported for mouse IgG subclass immunoglobulins (Hay, 1972; Greenberg, Shen and Roitt, 1973). This concentration was considered as the optimal level for 10^6 macrophages.

IgG2 immunoglobulins bound more efficiently than IgG1 and normal mouse immuno-

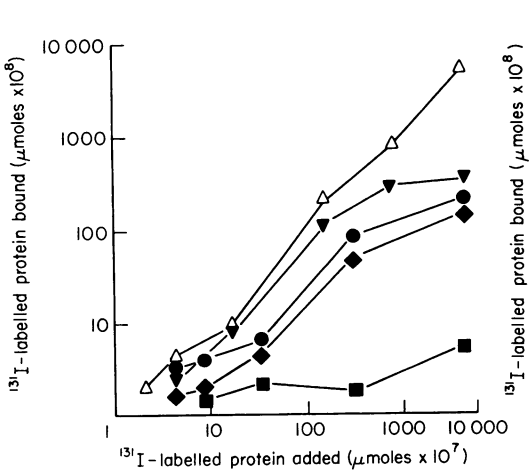

FIG. 2

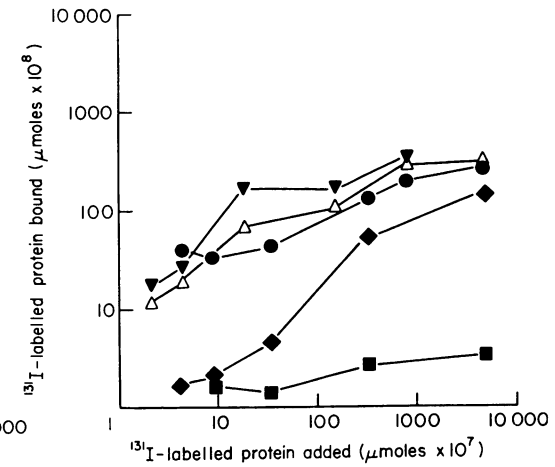

FIG. 3

FIG. 2. Binding of ^{131}I-labelled IgG1 (●), IgG1 Fc (▼) and IgG1 pFc′ (△) to 10^6 mouse peritoneal macrophages. NMIg (◆) and Fab (■) are the same as in Fig. 1.

FIG. 3. Binding of ^{131}I-labelled IgG2b, IgG2b Fc and IgG2b pFc′ to 10^6 mouse peritoneal macrophages, NMIg and Fab are the same as in Fig. 1.

globulin (positive control) and Fab did not bind. The relative order of binding observed was: IgG2b = IgG2a > IgG1 > NMIg ≫ Fab.

In both IgG1 and IgG2b, Fc and pFc′ were found to be better than the whole molecule in binding to macrophages (Figs 2 and 3). This effect was most obvious with IgG1 pFc′ at concentrations above 300×10^{-7} μmoles per 10^6 macrophages (Fig. 2).

INHIBITION OF BINDING OF IgG1 AND IgG2b

In the inhibition of IgG1 with IgG1 Fc and IgG1 pFc′, in contrast to the better binding observed in the direct binding experiments, no significant differences were found compared to IgG1 (Fig. 4b). But in the case of IgG2b, concentration dependent inhibitions were obtained with IgG2b Fc and IgG2b pFc′, which paralleled the direct binding patterns (Fig. 4a).

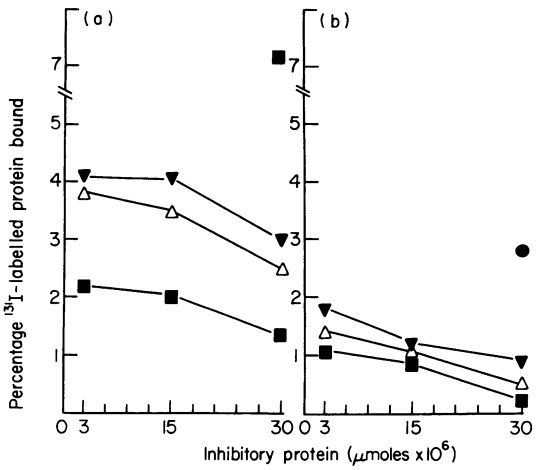

FIG. 4. (a) Inhibition of macrophage binding of ^{131}I-labelled IgG2b with unlabelled IgG2b, IgG2b Fc and IgG2b pFc′. (■) Binding of ^{131}I-labelled IgG2b in the absence of inhibitory protein; (▼) Inhibition with IgG2b pFc′; (△) inhibition with IgG2b Fc; (■) inhibition with IgG2b. (b) inhibition of macrophage binding of ^{131}I-labelled IgG1 with unlabelled IgG1, IgG1 Fc and IgG1 pFc′; (●) binding of ^{131}I-labelled IgG1 in the absence of inhibitory protein; (▼) inhibition with IgG1 pFc′; (△) inhibition with IgG1 Fc; (■) inhibition with IgG1.

CYTOPHILIC ROSETTE INHIBITION

Inhibition of cytophilic rosettes was performed with the Fc and pFc′ fragments of IgG1 and IgG2b, over a concentration range of $0{\cdot}5\text{–}2{\cdot}13 \times 10^{-5}$ μmoles per 10^6 macrophages. In both cases, Fc and pFc′ were found to be better than the whole immunoglobulin. But in contrast to the direct binding patterns, IgG1 pFc′ was much less efficient than IgG2b pFc′ in inhibiting cytophilic rosettes (Fig. 5). IgG1 pFc′ showed a 42 per cent inhibition at a concentration of $2{\cdot}13 \times 10^{-5}$ μmoles per 10^6 macrophages and dropped to 16 per cent at $0{\cdot}5 \times 10^{-5}$ μmoles. With IgG2b pFc′ this variation was from 86 to 62 per cent. At the highest concentration studied ($2{\cdot}13 \times 10^{-5}$ μmoles per 10^6 macrophages) IgG1 showed a very low inhibition of 8 per cent in contrast to the 73 per cent inhibition by IgG2b.

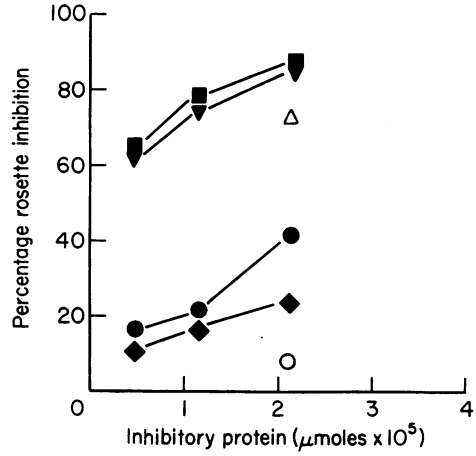

FIG. 5. Inhibition of mouse macrophage cytophilic rosettes with IgG1 (○), IgG1 Fc (◆), IgG1 pFc'(●), IgG2b (△), IgG2b Fc (■) and IgG2b pFc' (▼).

DISSOCIABILITY OF THE BOUND IMMUNOGLOBULINS

The binding of cytophilic antibodies to macrophages is weak and reversible and the bound immunoglobulin can be eluted from the macrophage in a subsequent incubation with the medium (Berken and Benacerraf, 1966; Phillips-Quagliata, Levine, Quagliata and Uhr, 1971; Tizard, 1972). The extent of elution of macrophage bound IgG1, IgG2a and IgG2b was determined in order to compare the relative binding affinities.

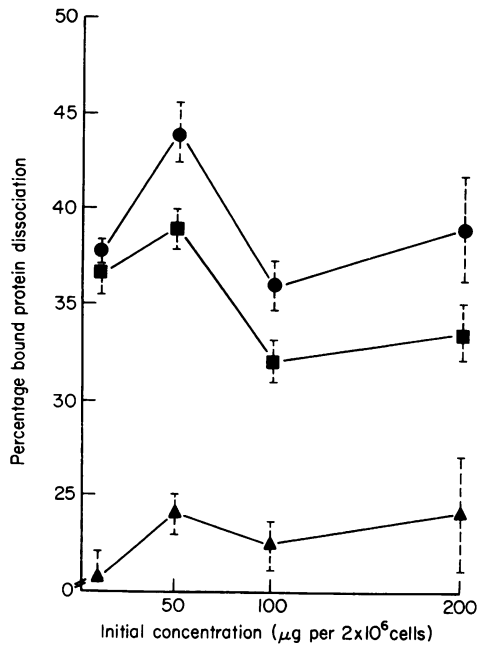

FIG. 6. Dissociability of ^{131}I-labelled immunoglobulin bound to macrophages after incubation of 37° for 1 hour. The results are expressed as percentage dissociation of bound IgG1 (●), IgG2a (▲) and IgG2b (■) relative to the amounts bound in the control experiments.

Macrophages were incubated with labelled IgG1, IgG2a and IgG2b at saturation levels (1–200 μg per 2×10^6 macrophages), at 4° for 1 hour. The washed cells were then incubated for a further hour at 37° in immunoglobulin-free medium, washed with five changes of medium, and counted. In the controls, the cells were counted after the first incubation. The amount of bound protein lost due to the second incubation was calculated and expressed as the percentage of the amount bound in the control experiments. The results of three different experiments with the same preparation of myeloma protein are given in Fig. 6.

Some of the immunoglobulins bound to macrophages were eluted when incubated at 37° in immunoglobulin-free medium. The percentage loss was highest for IgG1, demonstrating its weaker affinity.

DISCUSSION

We have demonstrated cytophilic activity in the mouse IgG subclasses by both direct binding and rosette inhibition. In all the experiments IgG2 was more cytophilic than IgG1. However, IgG1 can clearly bind at the higher concentrations when not in competition with IgG2. Therefore, it would not be correct to conclude that IgG1 is completely non-cytophilic, but in physiological conditions in competition with IgG2 it probably binds in very low amounts relative to its high serum concentration. Our studies of macrophage-bound IgG1, IgG2a and IgG2b have shown that IgG1 is the most easily eluted immunoglobulin.

The Fc and pFc′ fragments prepared from IgG2b were as active on a molar basis as the intact IgG2b molecule. This, taken together with the inability of Fab to bind to macrophages, allows us to conclude that the binding of IgG is a function of Fc, and in particular the C_H3 region. Berken and Benacerraf (1966) examined guinea-pig cytophilic antibodies and suggested that the binding site for macrophages was in the Fc region. Also, isolated Fc could inhibit the binding of human monocytes to red cells coated with anti-D (LoBuglio, Cotran and Jandl, 1967). Some slight inhibitory activity has been demonstrated in $F(ab')_2$ fragments (Huber *et al.*, 1971; Abramson, Gelfand, Jandl and Rosen, 1970) suggesting that the binding site might be in the C_H2 region, although the possibility of contaminating IgG or Fab/c fragments could not be ruled out. In contrast, MacLennan, Connell and Gotch (1974) were unable to inhibit neutrophil phagocytosis of sensitized bacteria with immune complexes of antigen and the Facb fragment of rabbit IgG. Since this fragment contains both the Fab regions and the C_H2 domain, this suggests that the C_H3 domain may be the site for cell binding. In a direct but heterologous system, Yasmeen *et al.* (1973) have shown that red cells coated with human pFc′ or Fc fragments could bind to guinea-pig macrophages. Recently we have demonstrated that human pFc′ fragment is capable of inhibiting the binding of human anti-D-coated red cells (IgG antibody) to human monocytes (Okafor *et al.*, 1974). As a control in this system, pFc′ fragments of each of the different IgG subclasses were used and fragments from the IgG1 and IgG3 subclasses possessed the greater inhibitory activity, in agreement with the association of this biological activity with these subclasses of whole IgG (Hay *et al.*, 1972). Ramasamy, Secher and Adetugbo (1975) have shown that a mutant myeloma protein with a deletion of the C_H3 region failed to inhibit Fc rosette formation with mouse lymphocytes while the original myeloma protein with an intact C_H3 region gave good inhibition.

In summary, this present study provides evidence in favour of: (1) in mouse cytophilic antibodies, the macrophage binding site is located in the C_H3 region; (2) Edelman's

'domain hypothesis' (Edelman, Cunningham, Gall, Gottelieb, Rutishauser and Waxdal, 1969; Edelman, 1970), which suggests that the polypeptide chains of IgG are folded into six discrete globular regions, each evolved for a particular biological activity.

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REFERENCES

ABRAMSON, N., GELFAND, E. W., JANDL, J. H. and ROSEN, F. S. (1970). 'The interactions between human monocytes and red cells. Specificity for IgG subclasses and IgG fragments.' *J. exp. Med.*, 1207.

BERKEN, A. and BENACERRAF, B. (1966). 'Properties of antibodies cytophilic for macrophages.' *J. exp. Med.*, **123,** 119.

BOYDEN, S. V. and SORKIN, E. (1960). 'The adsorption of antigen by spleen cells previously treated with antiserum *in vitro*.' *Immunology*, **3,** 272.

BOYDEN, S. V. and SORKIN, E. (1961). 'The adsorption of antibody and antigen by spleen cells *in vitro*. Some further experiments.' *Immunology*, **4,** 244.

BOYDEN, S. V. (1964). 'Cytophilic antibody in guinea pigs with delayed-type hypersensitivity.' *Immunology*, **7,** 474.

DISSANAYAKE, S. and HAY, F. C. (1975). 'Pepsin digestion of mouse IgG immunoglobulins, subfragments of the Fc region.' *Immunochemistry*, **12,** 373.

EDELMAN, G. M. (1970). 'The covalent structure of a human γG immunoglobulin. XI. Functional implications.' *Biochemistry*, **9,** 3197.

EDELMAN, G. M., CUNNINGHAM, B. A., GALL, W. E., GOTTELIEB, P. D., RUTISHAUSER, U. and WAXDAL, M. J. (1969). 'The covalent structure of an entire γG immunoglobulin molecule.' *Proc. nat. Acad. Sci. (Wash.)*, **63,** 78.

GREENBERG, A. G., SHEN, L. and ROITT, I. M. (1973). 'Characterization of the antibody-dependent cytotoxic cell. A non-phagocytic monocyte?' *Clin. exp. Immunol.*, **15,** 251.

GORINI, G., MEDGYESI, G. A. and DORIA, G. (1969). 'Heterogeneity of mouse myeloma γG-globulins as revealed by enzymatic proteolysis.' *J. Immunol.*, **103,** 1132.

HAY, F. C. (1972). *Biological properties of human and mouse immunoglobulin subclasses*. Ph.D. Thesis, Department of Immunology, The Middlesex Hospital Medical School, London W1.

HAY, F. C., TORRIGIANI, G. and ROITT, I. M. (1972). 'The binding of human IgG subclasses to human monocytes.' *Europ. J. Immunol.*, **2,** 257.

HOLM, G. (1974). Workshop report on 'Biological Function of the Fc fragment'. *Progress in Immunology II*, volume 1 (ed. by L. Brent and J. Holborow), p. 280. North-Holland, Amsterdam.

HUBER, H., DOUGLAS, S. D., NUSBACHER, J., KOCHWA, S. and ROSENFIELD, R. E. (1971). 'IgG subclass specificity of human monocyte receptor sites.' *Nature (Lond.)*, **229,** 419.

HUBER, H. and FUDENBERG, H. H. (1968). 'Receptor sites of human monocytes for IgG.' *Int. Arch. Allergy appl. Immunol.*, **34,** 18.

INCHLEY, C., GREY, H. M. and UHR, J. W. (1970). 'The cytophilic activity of human immunoglobulins.' *J. Immunol.*, **105,** 362.

LESLIE, R. G. Q. (1974). Workshop report on 'Biological Function of the Fc fragment'. *Progress in Immunology II*, volume 1 (ed. by L. Brent and J. Holborow), p. 280. North-Holland, Amsterdam.

LIEW, F. Y. (1971). 'The binding of the Fc fragment of guinea pig cytophilic antibody to peritoneal macrophages.' *Immunology*, **20,** 817.

LOBUGLIO, A. F., COTRAN, R. S. and JANDL, J. H. (1967). 'Red cells coated with immunoglobulin G. Binding and sphering by mononuclear cells in man.' *Science*, **158,** 1582.

MACLENNAN, I. C. M., CONNELL, G. E. and GOTCH, F. M. (1974). 'Effector activator 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.' *Immunology*, **26,** 303.

NELSON, D. S. and MILDENHALL, P. (1968). 'Studies on cytophilic antibodies. II. The production by guinea pigs of macrophage cytophilic antibodies to sheep erythrocytes and human serum albumin: relationship to the production of other antibodies and the development of delayed type hypersensitivity.' *Aust. J. exp. Biol. med. Sci.*, **46,** 33.

OKAFOR, G. O., TURNER, M. W. and HAY, F. C. (1974). 'Localization of monocyte binding site of human immunoglobulin G.' *Nature (Lond.)*, **248,** 228.

PARISH, W. E. (1965). 'Differentiation between cytophilic antibody and opsonin by a macrophage phagocytic system.' *Nature (Lond.)*, **208,** 594.

PHILLIPS-QUAGLIATA, J. M., LEVINE, B. B., QUAGLIATA, F. and UHR, J. W. (1971). 'Mechanisms underlying binding of immune complexes to macrophages.' *J. exp. Med.*, **133,** 589.

RAMASAMY, R., SECHER, D. S. and ADETUGBO, K. (1975). 'C_H3 domain of IgG as binding site to Fc receptor on mouse lymphocytes.' *Nature (Lond.)*, **253,** 656.

TIZARD, I. R. (1972). 'Elution of macrophage bound immunoglobulin by temperature changes *in vitro*.' *Immunology*, **22,** 69.

TORRIGIANI, G. (1972). 'Quantitative estimation of antibodies in the immunoglobulin classes of the mouse. Effect of adjuvants on the antibody response to human serum albumin and keyhole limpet haemocyanin.' *Clin. exp. Immunol.*, **11,** 125.

YASMEEN, D., ELLERSON, J. R., DORRINGTON, K. J. and PAINTER, R. H. (1973). 'Evidence for the domain hypothesis: location of the site of cytophilic activity toward guinea pig macrophages in the C_H3 homology region of human immunoglobulin G.' *J. Immunol.*, **110,** 1706.