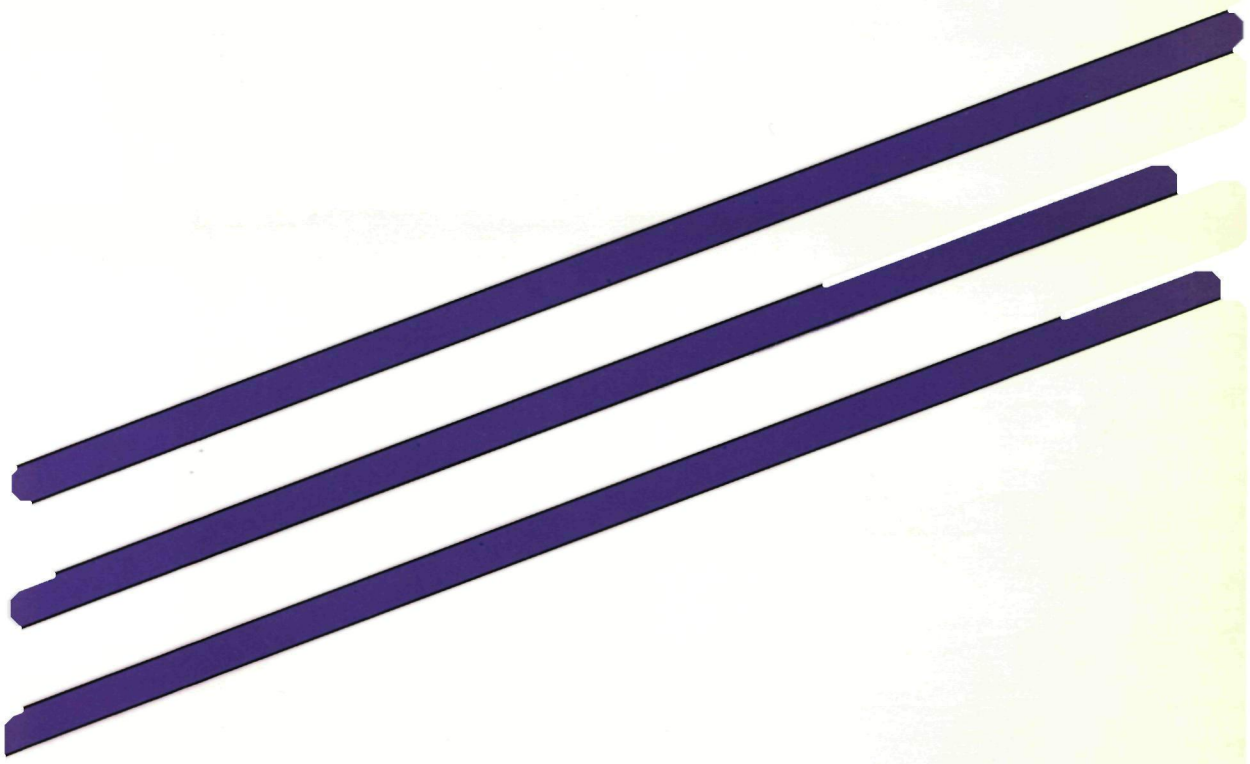
HUMAN MONOCYTE Fcγ RECEPTORS



J.G.J. van de Winkel

HUMAN MONOCYTE Fcγ RECEPTORS

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EEN WETENSCHAPPELIJKE PROEVE OP HET
GEBIED VAN DE GENEESKUNDE EN TANDHEELKUNDE

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Promotores: Prof.dr. R.A.P. Koene
Prof.dr. P.J.A. Capel (RU Utrecht)

Co-referent: Dr. W.J.M. Tax

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Aan Linda
Aan mijn ouders

CONTENTS

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
B-CLL	chronic lymphocytic leukemia of B origin
BSA	bovine serum albumin
CD	cluster of differentiation
DNP	dinitrophenyl
EA	antibody-sensitized erythrocytes
FcγR	receptor(s) for the Fc portion of IgG
FCS	foetal calf serum
h	human
HR	high-responder
HRBC	human erythrocytes
Ig	immunoglobulin
LR	low-responder
m	mouse
mAb	monoclonal antibody(ies)
n	number of individual experiments
PEG	polyethylene glycol
PBS	phosphate-buffered saline

CHAPTER 1

INTRODUCTION

Several types of cell participate together with immunoglobulins and a variety of other soluble molecules in the defense system of organisms. Immunoglobulins are bifunctional molecules, consisting of a part involved in recognition and binding to antigens, and another part, the Fc moiety, which is essential for their biological function. A special group of membrane receptors which can interact with this Fc part of immunoglobulins, Fc receptors, provide an important link between humoral and cellular aspects of immunity. In this way antigen specificity (provided by antibody) is coupled to the effector mechanisms of Fc receptor-positive cells that by themselves are not antigen-specific.

The existence of this type of receptor was first reported in the early sixties (1-3), and the term Fc receptor was suggested by Paraskevas et al. in 1971 (4). Fc receptors have a widespread distribution. They are found on all types of lymphocytes, on monocytes, macrophages, granulocytes, mast cells, and platelets. Furthermore, they are expressed on a variety of endothelial and epithelial cells and even on several types of parasites and bacteria. The induction of "Fc receptor-like" structures on cells after infection by viruses (5,6), and certain strains of mycoplasm (7) is also well known. Interaction of the Fc portion of an antibody with an Fc receptor can induce a variety of biological effects, e.g. phagocytosis of opsonized particulate antigens, clearance of immune complexes, degranulation, antibody-dependent cell-mediated cytotoxicity, release of inflammatory mediators (such as prostaglandins and hydrolytic enzymes), generation of reactive oxygen species, and regulation of immunoglobulin synthesis (for reviews see 8-14).

Fc receptors may also be clinically relevant. The expression or function of Fc receptors has been reported to be abnormal in a number of human diseases. These include malaria (15), leprosy (16), autoimmune diseases such as systemic lupus erythematosus (17), and

different types of cancer (18). Detailed knowledge about Fc receptors may furthermore be essential in view of the increased interest in the application of antibodies in clinical therapy and because previous studies have demonstrated that $F(ab')_2$ fragments of antibodies were therapeutically ineffective (19,20). Since the introduction of the hybridoma technique by Köhler and Milstein in 1975 (21), an increasing number of mouse monoclonal antibodies has been applied for immunotherapy in humans. They have been used in the therapy of cancer (22-24), immunosuppressive therapies after organ transplantation (25,26), and also in the treatment of autoimmune diseases such as autoimmune thrombocytopenic purpura (27). As human complement usually fails to lyse cells coated with mouse monoclonals (28), cellular effector mechanisms are probably important. Mouse antibodies bind poorly to human granulocytes (29), and cells of the mononuclear phagocyte system composed of monoblasts,promonocytes, monocytes, and macrophages (30) may therefore be the most important effector cells. Interestingly, monocyte-derived macrophages have been found to interact well with mouse anti-tumor antibodies, and were furthermore able to destroy sensitized human cancer cells (31).

Mammalian immunoglobulins are at present divided into five classes, designated IgG, IgA, IgE, IgM, and IgD, which are characterized by specific antigenic determinants in their heavy chains. Immunoglobulin G, which is quantitatively the major serum immunoglobulin and therapeutically the most interesting one, can furthermore be divided into several antigenically distinct subclasses. In humans these are named IgG1, IgG2, IgG3, and IgG4, whereas in the mouse, IgG subclasses are called IgG1, IgG2a, IgG2b, and IgG3. Receptors which interact with the Fc moiety of IgG, FcγR, on mononuclear phagocytes have been studied most intensively in the mouse system. Knowledge of mouse FcγR has progressed very well, both by the availability of purified immunoglobulins of the different mouse IgG subclasses and by the development of monoclonal anti-FcγR antibodies. Three different types of FcγR are currently recognized on mouse macrophages:

FcγRI, which binds monomeric mouse (m)IgG2a with high affinity, FcγRII, which interacts with aggregated or complexed antibodies of the mIgG1 and mIgG2b isotypes, and finally a third type of FcγR, FcγRIII, which interacts with mIgG3 antibodies in immune complex form. Beside differences in ligand-binding specificity, these receptors are also differentially sensitive to the protease trypsin. FcγRI is trypsin-sensitive, whereas binding to FcγRII and III are not affected by this enzyme, a finding of considerable experimental importance (for reviews see 9,14,32). Furthermore, these types of FcγR have been observed to be modulated independently, both in vitro and in vivo, which demonstrates that they are independently organized in the cell membrane (33).

In May 1985, when we started these investigations, only a single class of FcγR on human monocytes had unequivocally been demonstrated, a sialoglycoprotein with a molecular weight of 72 kDa (34), which interacts with high affinity ($Ka=10^8-10^9 M^{-1}$) with human IgG. Although all four human (h) IgG subclasses are bound by this receptor, their affinity was found to vary considerably. The subclasses hIgG1 and hIgG3 are bound much more efficiently than either hIgG2 or hIgG4 (35,36). On human peritoneal macrophages the existence of more than one type of FcγR was suggested from binding studies with hIgG1 dimers (37). Evidence for a comparable multiplicity of FcγR on human monocytes was derived from a quite different type of experiments. In these studies the induction of human T cell proliferation by mouse anti-CD3 antibodies in mononuclear cell suspensions was analyzed. Cross-linking of CD3 antigens, via anti-CD3 antibodies, by human monocyte FcγR was found to be essential for T cell mitogenesis (see Fig.1). By this type of experiments, human FcγR can be detected, which can functionally interact with mouse IgG, resulting in T cell proliferation (for review see 38). Monocytes from essentially all individuals supported induction of T cell proliferation by mIgG2a antibodies, whereas induction of T cell mitogenesis by mIgG1 antibodies was mediated only by monocytes from about 70% of Caucasian individuals (39). T cell proliferation

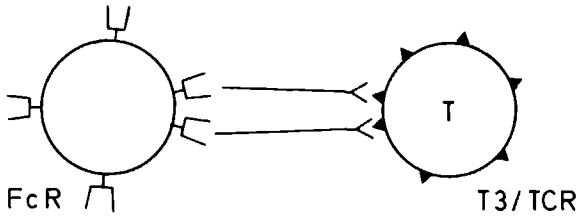

Fig.1. Antibodies against CD 3/T cell receptor can induce resting T cells to proliferate. Fc receptor positive cells (e.g. monocytes) are essential in this process.

induced by mIgG2a antibodies was susceptible to inhibition by monomeric hIgG and heat-aggregated mIgG2a, while mIgG1-induced mitogenesis could only be inhibited by aggregated mIgG1 and not by either hIgG or aggregated mIgG2a (40,41). It was furthermore observed that monocytes from individuals which supported the mIgG1-induced T cell proliferation efficiently (high-responder individuals; HR) also formed rosettes with mouse IgG1-sensitized erythrocytes, whereas monocytes from the other donors (low-responder individuals; LR) failed to do so (41). These data strongly suggested the presence of two different types of FcγR on human monocytes, one of them (the receptor which interacts with mouse IgG1) being polymorphic. The physiological functioning of both FcγR appeared mysterious. One of the receptors has an extremely high affinity for hIgG ($Ka=10^8-10^9$ M^{-1}) and would be expected to be saturated continuously with hIgG, which is present at approximately $10^{-5}M$ in human serum. The other FcγR seems to interact efficiently with mIgG1 antibodies, but not with hIgG.

The objective of this thesis was to study the characteristics, interrelation and function of the different types of FcγR on human monocytes. More specifically, we searched for an answer to the following questions:

- What is the specificity of both FcγR with respect to different subclasses of human and mouse IgG?

- What is the affinity of both types of FcγR for their respective ligands?

- Are these FcγR indeed independent membrane structures (or, alternatively, is there a single structure with two independent binding sites)?

- Are both FcγR functionally active (e.g. in antibody-dependent cell-mediated cytotoxicity)?

- Are the two types of FcγR different with respect to protease-sensitivity?

- What is the physiological significance of the FcγR which can interact with mouse IgG1 but apparently not with human IgG?

REFERENCES

1. Boyden, S.V., and Sorkin, E. The adsorption of antigen by spleen cells previously treated with antiserum in vitro. Immunology 1960; 3: 272-283.
2. Howard, J.G., and Benacerraf, B. Properties of macrophage receptors for cytophilic antibodies. Brit.J.Exp.Pathol. 1966; 47: 193-200.
3. Berken, A., and Benacerraf, B. Properties of antibodies cytophilic for macrophages. J.Exp.Med. 1966; 123: 119-144.
4. Paraskevas, F., Lee, S.-T., Orr, K.B., and Israels, L.G. Reverse immune cytoadherence: A technique for the detection of surface-associated γ-globulins on lymphoid cells. J.Immunol.Methods 1971; 1:1-17.
5. Rahman, A.A., Teschner, M., Sethi, K.K., and Brandis, H. Appearance of IgG (Fc) receptor(s) on cultured human fibroblasts infected with human cytomegalovirus. J.Immunol. 1976; 117: 253-258.
6. Para, M.F., Baucke, R.B., and Spear, P.G. Immunoglobulin G(Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. J.Virol. 1980; 34: 512-520.
7. Lemke, H., Krausse, R., Lorenzen, J., and Havsteen, B. Mycoplasma infection of cell lines can stimulate the expression of Fc receptors by binding of the carbohydrate moiety of antibodies. Eur.J.Immunol. 1985; 15: 442-447.
8. Dickler, H.B. Lymphocyte receptors for immunoglobulin. Adv.Immunol. 1976; 24: 167-214.
9. Unkeless, J.C., Fleit, H., and Mellman, I.S. Structural aspects and heterogeneity of immunoglobulin Fc receptors. Adv.Immunol. 1981; 31: 247-270.
10. Kerbel, R.S., and Elliott, B.E. Detection of Fc receptors. Methods Enzymol. 1983; 93: 113-147.
11. Burton, D.R. Immunoglobulin G: Functional sites. Molec. Immun. 1985; 22: 161-206.
12. Anderson, C.L., and Looney, R.J. Human leucocyte IgG Fc receptors. Immunol.Today 1986; 7: 264-266.
13. Morgan, E.L., and Weigle, W.O. Biological activities residing in the Fc region of immunoglobulin. Adv.Immunol. 1987; 40: 61-134.
14. Unkeless, J.C., Scigliano, E., and Freedman, V.H. Structure and function of human and murine receptors for IgG. Ann. Rev.Immunol. 1988; 6: 251-626.
15. Ward, K.N., Warrell, M.J., Rhodes, J., Looareesuwan, S., and White, N.J. Altered expression of human monocyte Fc receptors in plasmodium falciparum malaria. Infect.Immun. 1984; 44: 623-626.
16. Mistry, N.F., Birdi, T.J., Mahadevan, P.R., and Antia, N.H. Mycobacterium leprae-induced alterations in macrophage Fc receptor expression and monocyte-lymphocyte interaction in familial contacts of leprosy patients. Scand.J.Immunol. 1985; 22: 415-423.

17. Frank, M.M., Lawley, T.J., Hamburger, M.I., and Brown, E.J. Immunoglobulin G Fc receptor-mediated clearance in autoimmune diseases. Ann.Intern.Med. 1983; 98: 206-218.
18. Rhodes, J. Altered expression of human monocyte Fc receptors in malignant disease. Nature 1977; 265: 253-255.
19. Mandel, M.A., and Asofsky, R. The effects of heterologous anti-thymocyte sera in mice. II. Quantitative evaluation of IgM, IgG, and their subunits in suppressing homograft reactions. J.Immunol. 1968; 100: 1259-1267.
20. Lance, E.M., Medawar, P.B., and Taub, R.N. Antilymphocyte serum. Adv.Immunol. 1973; 17: 1-92.
21. Köhler, G., and Milstein, C. Continuous cultures of fused cells secreting antibody of pre-defined specificity. Nature 1975; 256: 495-497.
22. Miller, R.A., Maloney, D.G., Warnke, R., and Levy, R. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. N.Engl.J.Med. 1982; 306: 517-522.
23. Sears, H.F., Herlyn, D., Steplewski, Z., and Koprowski, H. Effects of monoclonal antibody immunotherapy on patients with gastrointestinal adenocarcinoma. J.Biol.Resp.Modif. 1984; 3: 138-150.
24. Capel, P.J.A., Preijers, F.W.M.B., Allebes, W.A., and Haanen, C. Treatment of chronic lymphocytic leukaemia with monoclonal anti-idiotypic antibody. Neth.J.Med. 1985; 28: 112-118.
25. Ortho Multicenter Transplant Study Group. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. N.Engl.J.Med. 1985; 313: 337-342.
26. Tax, W.J.M., Van de Heijden, H.W., Willems, H.W., Hoitsma, A.J., Berden, J.H.M., Capel, P.J.A., and Koene, R.A.P. Immunosuppression with monoclonal anti-T3 antibody (WT32) in renal transplantation. Transpl.Proc. 1987; 19: 1905-1907.
27. Clarkson, S.B., Bussel, J.B., Kimberly, R.P., Valinsky, J.E., Nachman, R.L., and Unkeless, J.C. Treatment of refractory immune thrombocytopenic purpura with an anti-Fcγ-receptor antibody. N.Engl.J.Med. 1986; 314: 1236-1239.
28. Ritz, J., and Schlossman, S.F. Utilization of monoclonal antibodies in the treatment of leukemia and lymphoma. Blood 1982; 59:1-11.
29. Roberton, D.M., Georgiou, G.M., Ellis, W.M., and Hosking, C.S. Murine anti-lymphocyte monoconal antibodies fail to act as opsonins for allogeneic human peripheral blood polymorphonuclear phagocytes. Clin.Exp.Immunol. 1984; 55: 444-450.
30. Nathan, C.F., Murray, H.W., and Cohn, Z.A. The macrophage as an effector cell. N.Engl.J.Med. 1980; 303: 622-626.
31. Steplewski, Z., Lubeck, M.D., and Koprowski, H. Human macrophages armed with murine immunoglobulin G2a antibodies to tumors destroy human cancer cells. Science 1983; 221: 865-867.
32. Unkeless, J.C., and Wright, S.D. Structure and modulation of Fc and complement receptors. Top.Immunobiol. 1984; 14: 171-187.
33. Ezekowitz, R.A.B., Bampton, M., and Gordon, S. Macrophage activation selectively enhances expression of Fc receptors for IgG2a. J.Exp.Med. 1983; 157: 807-812.

34. Anderson, C.L. Isolation of the receptor for IgG from a human monocyte cell line (U937) and from human peripheral blood monocytes. J.Exp.Med. 1982; 156: 1794-1805.
35. Anderson, C.L., and Abraham, G.N. Characterization of the Fc receptor for IgG on a human macrophage cell line, U937. J.Immunol. 1980; 125: 2735-2741.
36. Kurlander, R.J., and Batker, J. The binding of human immunoglobulin G1 monomer and small, covalently cross-linked polymers of immunoglobulin G1 to human peripheral blood monocytes and polymorphonuclear leukocytes. J.Clin.Invest. 1982; 69: 1-8.
37. Kurlander, R.J., Haney, A.F., and Gartrell, J. Human peritoneal macrophages possess two populations of IgG Fc receptors. Cell. Immunol. 1984; 86: 479-490.
38. Chang, T.W. Regulation of immune response by antibodies: the importance of antibody and monocyte Fc receptor interaction in T-cell activation. Immunol.Today 1985; 6: 245-249.
39. Tax, W.J.M., Willems, H.W., Reekers, P.P.M., Capel, P.J.A., and Koene, R.A.P. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. Nature 1983; 304: 445-447.
40. Looney, R.J., and Abraham, G.N. The Fc portion of intact IgG blocks stimulation of human PBMC by anti-T3. J.Immunol. 1984; 133: 154-156.
41. Tax, W.J.M., Hermes, F.F.M., Willems, H.W., Capel, P.J.A., and Koene, R.A.P. Fc receptors for mouse IgG1 on human monocytes: polymorphism and role in antibody-induced T cell proliferation. J.Immunol. 1984; 133: 1185-1189.

CHAPTER 2

A NEW PHOTOMETRIC METHOD FOR THE QUANTITATION OF Fc RECEPTORS FOR MURINE IgG1 ON HUMAN MONOCYTES AND CELL LINES

Jan G.J. van de Winkel, Wil J.M. Tax, Mieke C.J. van Bruggen, Kees E.P.van Roozendaal, Riki W. Willems, Hans L.P. van Duijnhoven, Peter J.A. Capel, and Robert A.P. Koene

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A new photometric method for the quantitation of Fc receptors for murine IgG1 on human monocytes and cell lines

J.G.J Van de Winkel [1], W J M. Tax [1], M C J. Van Bruggen [1], C.E.P. Van Roozendaal [1], H.W. Willems [2], J L.P Van Duijnhoven [1], P.J.A. Capel [1] and R.A.P Koene [1]

[1] *Department of Medicine, Division of Nephrology, and* [2] *Department of Pathology, St Radboud Hospital, University of Nijmegen, Nijmegen, The Netherlands*

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We have previously shown a polymorphism of human Fc receptors for mouse IgG1 using an EA rosette technique in which human erythrocytes sensitized with a murine IgG1 monoclonal antibody against glycophorin A acted as indicator cells We now describe a method to quantitate this EA rosetting using the pseudoperoxidase activity present in erythrocytes This photometric assay allows the sensitive quantitative determination of Fc receptor expression on human monocytes and cell lines Not only the human Fc receptor for murine IgG1 can be studied in this way, but the method can also be applied to other Fc receptors An important factor in this type of rosette assay appears to be the amount of negative charge present on the surface of the indicator erythrocytes. Using alcian blue as a probe, we found that this negative charge is higher on human erthyrocytes than on sheep erythrocytes, which may contribute to a better signal-to-noise ratio. The method described facilitates the characterization of Fc receptors and permits the rapid screening of monoclonal anti-Fc receptor antibodies

Key words Fc receptor, human, Monocyte, Cell line, Photometric assay, Pseudoperoxidase activity

Introduction

Membrane-associated receptors, recognizing the Fc portion of immunoglobulins (FcR), play important roles in the immune system (for review, see Unkeless et al., 1981) Because of the growing interest in the clinical use of murine monoclonal antibodies for immune therapy, it is important tc gain more insight into the structures on humar effector cells which can mediate interactions with murine immunoglobulins The interaction of mIgC with human FcR has been analyzed primarily ir studies on the monocyte-dependent induction o human T-cell proliferation by anti-CD3 mono clonal antibodies From these studies it was con cluded that virtually all individuals have monocyt FcR for mIgG2a and that the monocyte FcR fo mIgG1 (Tax et al., 1983, 1984), as well as th human FcR for mIgG2b antibodies (Tax et al 1985) are polymorphic.

There are various methods for studying FcI expression directly (reviewed by Kerbel et al 1983) Of these methods EA rosetting is a we established and sensitive assay to study FcR. Vis

Correspondence to J G J Van de Winkel Department of Medicine Division of Nephrology, St Radboud Hospital, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, The Netherlands

Abbreviations ASA, 5'-aminosalicylic acid, BSA, bovine serum albumin, FcR, receptor(s) for the Fc region of immunoglobulins, FCS, foetal calf serum, HRBC, human erythrocytes, IF, immunofluorescence, m, murine, OD, optical density, PBS, phosphate-buffered saline, RBC erythrocytes, SRBC, sheep erythrocytes

ual quantitation of EA rosettes however, is laborious In this report we describe a very sensitive photometric assay by which the FcR for mIgG1 (which is very difficult to study in direct assays (Tax et al , 1984)) on both monocytes and cell lines can be studied by FA rosetting We used the erythrocyte-associated pseudoperoxidase activity (Naumann, 1964) to quantitate the binding of human erythrocytes to monolayers of cells in microtitre plates The Fc dependency of the signal obtained was studied and furthermore it was shown that this assay is also suitable to study the expression of other Fc receptors such as that for human IgG

The present method can facilitate the characterization of FcR on human cells in different types of studies (e g , rosette inhibition studies, modulation experiments), and permits the rapid screening for anti-FcR antibodies

Materials and methods

Cell lines

Human myeloid (HL-60), monocytic (U937), erythromyeloid (K562), B-lymphoblastoid (SB), and T lymphoblastoid (Molt-4) cell lines were cultured in RPMI 1640 medium This medium was supplemented with 10% heat-inactivated FCS 2 mM glutamine, 1 mM sodium pyruvate, and gentamicin (50 μg/ml)

Peripheral blood monocytes

Mononuclear cells were isolated from buffy coats on a Percoll (Pharmacia, Uppsala, Sweden) gradient ($s = 1\,072$ g/ml) at 4° C Monocytes were purified from mononuclear cells by counterflow centrifugation in an elutriation system enabling continuous monitoring by flow cytometry as described by De Mulder et al (1981) The purity of the monocytes obtained was evaluated in cytocentrifuge preparations after staining for nonspecific esterase and with May-Grunwald-Giemsa The final suspensions consisted of at least 90% monocytes Viability of monocyte suspensions was assessed by trypan blue dye exclusion and exceeded 98% after elutriation

Since monocytes from different individuals show functional variability we considered it advantageous to have fixed and well-defined batches of these cells The purified monocytes were therefore cryopreserved as described by Shah et al (1984) and used later Both recovery and viability of cryopreserved monocytes were excellent (> 70% and > 95%, respectively)

Because of the polymorphism of monocyte FcR for mIgG1 antibodies, the accessory function of isolated monocytes was studied in mitogenesis assays as described by Tax et al (1983) Only monocytes which supported the proliferation of human T-lymphocytes, induced by murine anti-CD3 antibodies of the IgG1 subclass, were used for the studies described in this report

Comparison of two photometric assays for human erythrocytes

Erythrocytes can be quantitated using the absorbance of haemoglobin (Cooper et al , 1984) and also by the pseudoperoxidase-catalyzed conversion of a suitable substrate such as ASA (Naumann, 1964) 100 μl of serial dilutions of HRBC (in 50 mM phosphate buffer, pH 6 0) were dispensed into the wells of a flat-bottomed microtitre plate (Costar, Cambridge, MA) To half of the wells 100 μl of the same buffer was added, and after mixing the OD was determined in a Titertek Multiskan Readings were performed at 405 nm, the extinction maximum of erythrocyte-associated haemoglobin The other half of the wells was supplemented with 100 μl of 50 mM phosphate buffer, pH 6 0, containing 1 6 mg/ml ASA and 0 144% v/v H_2O_2 (final concentration) and incubated for 30 min at room temperature After mixing, the absorbance at 450 nm was measured Since the latter method proved to be more sensitive (see results section) we preferred this technique for the quantitation of EA rosettes

Sensitization of human erythrocytes

A 0 5% suspension of HRBC in PBS was incubated for 30 min at 37° C with various dilutions of antibody For mIgG1 EA rosetting we used ascites of an IgG1 monoclonal antibody against glycophorin A (a generous gift from M Bos, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) Human IgG EA rosetting was performed with Rhesus-positive HRBC and a poly-

clonal antiserum against Rhesus D (Merz&Dade, Dudingen, Switzerland) HRBC incubated with PBS alone served as controls for non-specific binding After incubation, sensitized (and, as a control, unsensitized) HRBC were washed, resuspended at 0 5% in RPMI 1640 containing 5% FCS, and used within 30 min

Microscopic EA rosette assay

200 μl of a monocyte suspension (2×10^6 cells/ml in RPMI 1640 with 5% FCS) and 50 μl of sensitized HRBC suspension were mixed, centrifuged (4 min at $20 \times g$), and incubated for 1 h at 4°C Monocytes with at least three bound HRBC were scored as rosettes, using a microscope with 400 × final magnification

Photometric EA rosette assay

First, monolayers of either monocytes or cell lines were prepared With monocytes, 100 μl of a cell suspension (4×10^6 cells/ml in RPMI 1640 with 10% FCS) were dispensed into individual wells of a 96-well flat-bottomed microtitre plate and incubated for 1 h at 37°C Non-adherent cells were removed by one wash in warm PBS (37°C), in which the plates were immersed vertically and moved back and forth three times

In order to obtain monolayers of cell lines, these cells were coated to microtitre plates Polystyrene microtitre plates were precoated with poly-l-lysine (0 1 mg/ml in PBS) for at least 1 h at room temperature After three washes with PBS, 100 μl of a cell suspension (4×10^6 cells/ml in RPMI 1640 with 10% FCS) were dispensed into each well and the plate incubated at room temperature for 1 h After centrifugation (5 min at $300 \times g$), non-adherent cells were washed away as described above

To each well, containing a monolayer of either monocytes or a cell line, 100 μl of a 0 5% suspension of sensitized HRBC were added and incubated for 1 h at 4°C After this incubation, the plates were washed thrice by immersion in warm PBS as described above To each well 200 μl of a freshly prepared substrate solution, 0 8 mg/ml ASA dissolved in 50 mM phosphate buffer, pH 6 0, containing 0 072% v/v H_2O_2 was added After 30 min, extinction was measured at 450 nm in a Titertek Multiskan

To correct for variation in background absorbance values between experiments a binding index was calculated for each experiment as follows

$$[1 - (E_{450}/EA_{450})] \times 100 = \text{binding index}$$

where E_{450} is the extinction at 450 nm in wells to which HRBC *without* sensitizing antibody were added, and EA_{450} the extinction at 450 nm in wells to which *sensitized* HRBC were added

The HRBC suspensions sensitized with different dilutions of antiserum were always tested in duplicate The observed variation in binding indices was small (usually less than 10%) All experiments were performed at least three times and representative experiments are shown

$F(ab')_2$ fragments

Pepsin digestion of the monoclonal anti-glycophorin A antibody and subsequent isolation of the $F(ab')_2$ fragments was performed as described by Capel et al (1979) The purity of the fragments obtained was analyzed by SDS-PAGE

Immunofluorescence

The level of sensitization of HRBC with anti-glycophorin A antibody was evaluated by incubating 100 μl of a 1% suspension of sensitized HRBC in IF buffer (PBS containing 0 1% sodium azide and 1% BSA, Cohn Fraction V, Sigma, St Louis, MO) with 100 μl of a 1 50 dilution of (Fab fragments of) goat anti-mouse Ig (H&L)-FITC (Cappel, Malvern, PA) After incubating for 30 min at room temperature the cells were washed two times, fixed with 1% paraformaldehyde and subsequently analyzed by an Ortho 50-H flow cytometer In each sample the fluorescence intensity from 10000 cells was determined and the mean fluorescence intensity was calculated from the histograms

To test antigen-binding capacity of the $F(ab')_2$ fragments of IgG1 anti-glycophorin A, the inhibition of HRBC sensitization was studied in the following way To 50 μl of HRBC (1% suspension), 50 μl of $F(ab')_2$ fragments at a 1 50 dilution in IF buffer were added and incubated for 30 min at room temperature After this period 50 μl of intact anti-glycophorin A antibody, in various dilu-

tions were added to each suspension and incubated for another 30 min at room temperature The cells were then washed and incubated with 100 μl of subclass-specific goat anti-mouse IgG1-FITC (Nordic Tilburg The Netherlands) at a 1 50 dilution for 30 min (room temperature) After washing two times, the brightness of fluorescence was scored on a scale of negative to strongly positive on a Zeiss fluorescence microscope

Modulation of Fc receptors

Flat-bottomed microtitre plates were coated overnight with BSA (10 μg/ml in PBS) at 4°C After three washes either 100 μl PBS (control) or 100 μl of a rabbit anti-BSA antiserum (Dako, Copenhagen Denmark), at a saturating concentration as determined by ELISA, were added to each well for 1 h at room temperature The plates were washed thrice and 100 μl of a monocyte suspension (4×10^6 cells/ml in RPMI 1640 with 10% FCS) were added to each well and the plates incubated for 90 min at 37°C After this incubation non-adherent cells were washed away and FcR expression determined photometrically as described above

Quantitation of anionic sites on erythrocytes

The number of anionic sites on RBC was measured by studying the binding of alcian blue 8GX (Sigma, St Louis, MO) as has been described in detail by Levin et al (1985) RBC from three different species, man, sheep and rabbit, were washed and suspended in PBS at a final concentration of $1\,2 \times 10^9$ cells/ml 1 ml of the suspensions of RBC was mixed with 1 ml of alcian blue solution (at increasing concentrations) and incubated at 37°C for 30 min After removal of the cells by centrifugation the optical density of the supernatant was measured at 650 nm At this wavelength the optical density of alcian blue is linearly related to its concentration From the difference in optical density between the original solution and the supernatant after removal of the cells, the amount of alcian blue bound to the cells was determined

Results

Detection of erythrocytes by direct measurement of haemoglobin absorbance and by quantitation of haemoglobin catalyzed pseudoperoxidase activity

Before we studied the binding of sensitized HRBC to human cells, we first investigated the detection of HRBC by different photometric methods In Fig 1 the haemoglobin absorbance of HRBC and the HRBC-associated pseudoperoxidase activity are shown In preliminary experiments with graded numbers of HRBC optimal conditions for measuring the pseudoperoxidase activity had been determined In Fig 1 it is shown that by using the pseudoperoxidase activity a much higher sensitivity of HRBC detection can be reached compared with direct measurement of haemoglobin absorbance The threshold for detection of HRBC is about ten-fold lower when using HRBC-associated pseudoperoxidase activity and the slope of the curve is also much steeper than when haemoglobin absorbance is used for the detection of HRBC A linear response was obtained up to 8×10^5 HRBC/well

Comparison of microscopic and photometric EA rosette assays for the detection of FcR for murine IgG1 on human monocytes

By changing the concentration of antibody used for sensitization of the erythrocytes the amount of

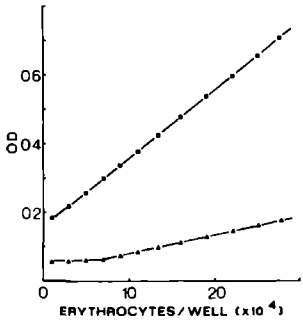

Fig 1 Comparison of measurement of haemoglobin absorbance directly (at 405 nm ▲) and after haemoglobin catalyzed conversion of ASA (at 450 nm ■) Extinction was determined in duplicate microtitre wells to which fixed numbers of HRBC were added

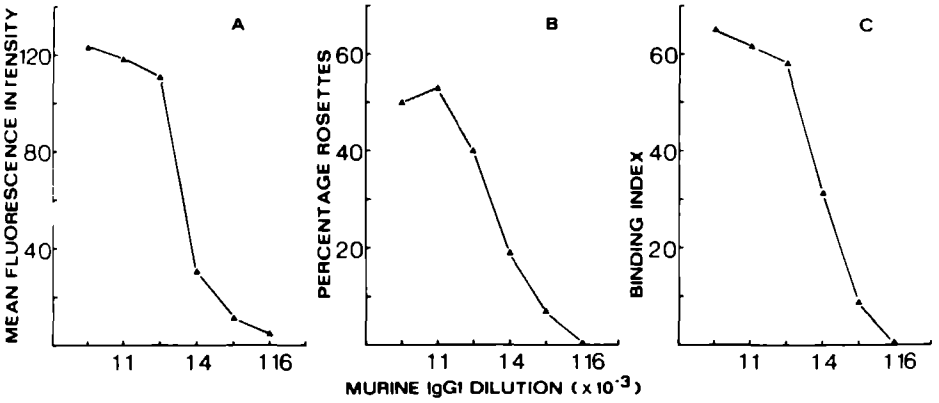

Fig 2 Comparison of EA IgG1 rosette formation by microscopic and photometric methods *A* Immunofluorescence of indicator HRBC sensitized with different dilutions of anti-glycophorin A monoclonal antibody *B* Percentage of EA rosettes *C* Binding indices in the photometric assay

bound IgG could be regulated When the dilution of sensitizing antibody was plotted against the amount of bound antibody (as reflected in the intensity of indirect immunofluorescence) a sigmoid curve was obtained (Fig 2*A*) There was a good correlation between the degree of sensitization of HRBC, and the percentage of EA rosettes either scored visually (Fig 2*B*) or photometrically (Fig 2*C*) In all the experiments with monocytes presented in this report we used 4×10^5 cells/well

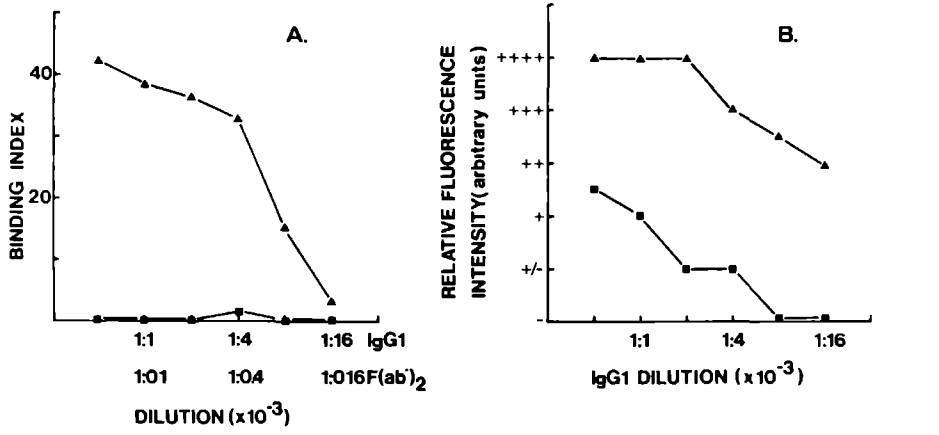

Fig 3 *A* Binding to human monocytes of HRBC which have been sensitized with intact IgG (▲) antibody directed against glycophorin A, and when sensitized with the $F(ab')_2$ fragments of the antibody (■) *B* Binding of intact IgG anti-glycophorin A to HRBC, as determined by immunofluorescence using FITC-labelled subclass-specific antibodies (▲), is inhibited when the HRBC have been preincubated with the $F(ab')_2$ fragments of the antibody (■)

It is noteworthy that at this cell density, direct measurement of haemoglobin absorbance (at 405 nm) was inadequate to quantitate rosetting

Fc dependency of signals obtained in the photometric EA rosette assay

In order to ascertain that the signals we obtained in the photometric assay of the FcR for mIgG1 were truly a manifestation of an interaction between FcR and the Fc portion of immunoglobulin molecules, we prepared $F(ab')_2$ fragments of the anti-glycophorin A antibody No residual IgG could be detected after pepsin digestion and purification of the $F(ab')_2$ fragments (not shown) When this preparation was used to sensitize HRBC no signal was found in the photometric EA rosette assay (Fig 3*A*) The $F(ab')_2$ fragments did bind to HRBC as seen by indirect fluorescence (data not shown), and were able to inhibit the binding of intact anti-glycophorin A antibody as illustrated in Fig 3*B* From these results we conclude that the signals obtained in the photometric EA rosette assay are indeed Fc-dependent

Assay of murine IgG1 FcR on human cell lines

Fig 4 shows that the photometric method is suitable for the assay of FcR for mIgG1 on human cell lines The level of FcR expression varies considerably between different lines Cell line Molt 4 gives no signal in the photometric assay whereas other cell lines exhibit binding of sensitized HRBC

Assay of human IgG FcR on human cell lines and monocytes

Above we showed that the photometric method can be used for studies of the FcR for mIgG1 Fig 5 illustrates that the 'classical'/high affinity FcR for human IgG can also be assayed by the photometric method In this assay we used a human antiserum against rhesus D to sensitize Rhesus D-positive HRBC When comparing Figs 4 and 5 it is apparent that the expression of FcR for mIgG1 on cell lines does not correlate in all instances with the expression of FcR for human IgG The human erythromyeloid cell line K562, for example, binds HRBC sensitized with human IgG poorly, whereas mIgG1-sensitized HRBC were bound very well by this cell line In contrast, cell line Molt-4 does not give any signal in either the assay for the human IgG FcR or for mIgG1 FcR The expression of the FcR for human IgG on monocytes is clearly positive and comparable to that on the cell lines U937 and HL-60 (Fig 5)

Modulation of FcR on human monocytes

Cell surface Fc receptors can be modulated when the cells are incubated with immune complexes coated to a solid support (Michl et al, 1979) In Fig 6 we show that FcR can be modulated from the apical surface of human monocytes In this experiment the human IgG FcR are

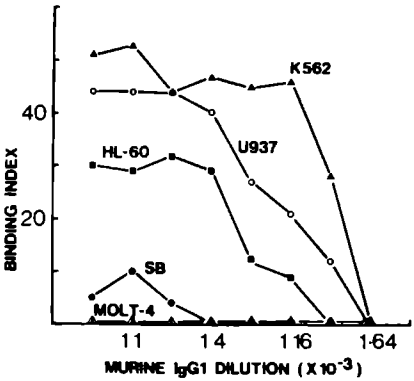

Fig 4 Binding of HRBC sensitized with mIgG1 antibody to human cell lines

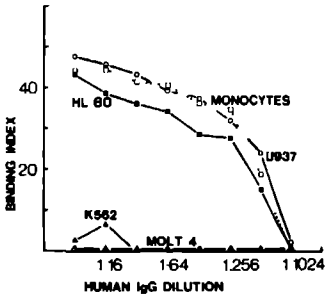

Fig 5 Binding of HRBC sensitized with human IgG to human monocytes (dashed line) and various cell lines

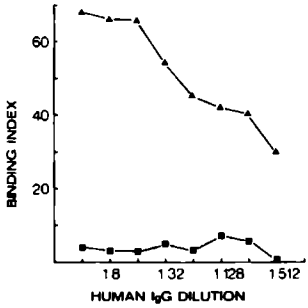

Fig 6 Modulation of FcR on human monocytes by immune complexes Monocytes were incubated on BSA coated microtitre plates only (▲) or on plates which had been coated with BSA and subsequently incubated with rabbit anti BSA antibody (■) FcR expression for human IgG was then determined photometrically

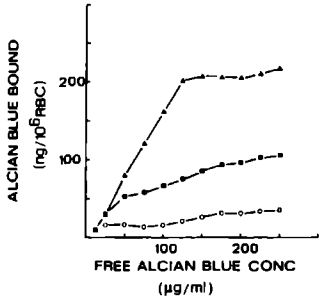

Fig 7 Alcian blue binding to erythrocytes from man (▲), sheep (■) and rabbits (○)

modulated by rabbit IgG, complexed to BSA This indicates that rabbit IgG can interact efficiently with the FcR for human IgG on human monocytes In similar experiments, using immobilized immune complexes containing mIgG1, we could modulate the monocyte FcR for mIgG1 (not shown) This data shows that the photometric assay can be applied to study the modulation of human Fc receptors

Negative charge of erythrocytes from different species

During our studies on human FcR interacting with mIgG of different isotypes, we observed that HRBC gave higher binding indices in the photometric assay and better reproducibility than SRBC The low binding indices found with SRBC were due to high non-specific binding of unsensitized erythrocytes to monolayers of both human monocytes and cell lines Since the negative charge on the RBC surface is an important factor in relation to its binding to other cells (e g , non-specific binding to cell lines), we studied whether there were profound differences between human, sheep, and rabbit erythrocytes with respect to this parameter Therefore, we measured the binding of alcian blue 8GX to different types of erythrocytes Fig 7 shows that HRBC can bind more alcian blue to their surface and therefore have a higher negative charge than SRBC Rabbit erythrocytes showed a very low binding of alcian blue

Discussion

Detection of FcR can be performed very sensitively by EA rosette assays Microscopic EA rosette assays are commonly used but require laborious visual counting procedures Furthermore, the problem of instability of rosettes interferes with the reliable evaluation of the percentage of FcR-positive cells Quantitation of EA rosette procedures can be performed sensitively and reproducibly using ^{51}Cr-labelled erythrocytes (Jungi, 1985) This method suffers from some disadvantages, such as time-consuming labelling procedures, shedding of label and problems of isotope handling Rummage and Leu (1985) described a method by which EA rosettes were quantitated by measuring the erythrocyte haemoglobin absorbance at 405 nm These authors used sensitized sheep erythrocytes to detect FcR on murine macrophage monolayers The sensitivity of their method was similar to that of ^{51}Cr-labelled erythrocyte assays A similar photometric method for the quantitation of erythrocyte phagocytosis in murine macrophages was described by Cooper et al (1984) Recently, Jungi (1985) reported the use of erythrocyte-associated pseudoperoxidase activity to quantitate the phagocytosis of sheep erythrocytes in human monocytes and macro-

phages This method was shown to be more sensitive than assays based on the use of ^{51}Cr-labelled erythrocytes A disadvantage of the method of Jungi is the fact that lysis of mononuclear cells is required Quantitation of erythrocytes in detergent lysates of phagocytes is prone to error, because of the endogenous peroxidase activity of monocytes Also, the presence of FcR on cell lines cannot be quantitated by this method since not all cell lines phagocytose the sensitized erythrocytes

In this report we present a new photometric method by which binding of Fc to different types of FcR on human monocytes and cell lines can be reproducibly quantitated with high sensitivity By measuring HRBC-associated pseudoperoxidase activity a much higher sensitivity was reached than by determination of haemoglobin absorbance directly The method reported by Rummage and Leu (1985), employing haemoglobin absorbance, proved to be too insensitive in the detection of FcR for mIgG1 on human monocytes Using our technique HRBC could be used for detecting between 1×10^4 and 8×10^5 erythrocytes/well with linear calibration curves This sensitivity matches that of the method described by Jungi (1985), but our method has the additional advantage that FcR on cell lines which do not actively phagocytose can also be studied Cell lines have been extremely valuable for our knowledge of the functional characteristics of FcR (Unkeless et al , 1981)

Sheep erythrocytes may not be the indicator erythrocytes of choice for EA rosette assays for two reasons First, unsensitized SRBC form rosettes with human T-cells which may be present in the cell suspensions to be studied Secondly, SRBC have a low negative surface charge, which could give rise to high non-specific binding of these erythrocytes We studied the negative surface charge on human, sheep, and rabbit erythrocytes by comparing the binding of the cationic dye alcian blue to these cells In most studies only the sialic acid content has been compared on erythrocytes from different species (Aminoff et al , 1976) The sialic acid content of the cell membrane, however, is not the only parameter responsible for the negative charge of cells, as was shown recently by Levin et al (1985) We found that SRBC bind significantly less alcian blue than HRBC, which indicates that they possess a much lower cell surface negative charge Rabbit erythrocytes did not bind much alcian blue, which is consistent with their low sialic acid content (Aminoff et al , 1976) The differences in alcian blue binding cannot be caused by the differences in surface area between the erythrocytes from different species (as can be calculated from the volumes of the different erythrocytes (Wessels and Veerkamp, 1973)) It is likely that the high, negative charge of HRBC contributed significantly to the positive results we obtained when using these erythrocytes as indicator cells in EA rosetting experiments

We have previously reported that FcR for mIgG1 can be detected by EA rosetting on monocytes from those individuals who exhibit a mitogenic response to mIgG1 anti-CD3 antibodies (Tax et al , 1984) We now show that the binding of IgG1 sensitized HRBC to such monocytes can be quantitated using the pseudoperoxidase activity of the erythrocytes Furthermore, with this method FcR for mIgG1 can also be demonstrated on cell lines K562, U937, and HL-60 Lemke et al (1985) could detect FcR for mIgG1 only on K562 and found furthermore a weak expression on HL-60 and no expression on U937, using sensitized sheep erythrocytes as indicator cells These results further accentuate the sensitivity of our indicator system

Our study suggests that the FcR for human IgG are present on the U937 and HL-60 cell lines, but are hardly detectable on the relatively undifferentiated K562 cell line When using indicator erythrocytes, sensitized with human IgG, we occasionally (at very low dilutions of sensitizing antiserum) observed a low degree of rosette formation with K562 This observation is in agreement with the work of other investigators, who showed that K562 cells possess low affinity FcR, while U937 and HL-60 cells have high affinity FcR specific for the Fc fragment of human IgG (McCool et al , 1985) Similar results were obtained by Anderson and Looney, as is evident from their recent review on the different types of FcR for human IgG (Anderson and Looney, 1986) Other authors (e g , Perussia et al , 1982), using indicator erythrocytes sensitized with rabbit antibody, observed a high degree of rosette formation with K562, an intermediate degree with U937 and a low degree with

HL-60. One explanation for this discrepancy could be that the FcR on K562 have a higher affinity for rabbit IgG than for human IgG (in contrast to FcR on human monocytes which have similar affinity for rabbit IgG and human IgG (Woof et al, 1986)). Furthermore, it is important to realize that the results from FcR assays are dependent on the sensitivity of the assay, and on the precise conditions employed

In conclusion, we describe a new, sensitive and straightforward FcR assay based on the quantitation of EA rosetting with human indicator erythrocytes, sensitized with human or murine IgG, by using the haemoglobin-catalyzed pseudoperoxidase activity. This method can be used for the characterization of FcR on human cells in different types of studies such as the modulation of FcR on immune complex-coated substrates or in rosette-inhibition studies (manuscript in preparation). The photometric assay should also be valuable for the screening of new anti-FcR monoclonal antibodies, which can then be used to study FcR in greater detail.

Acknowledgements

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References

Anderson, C L and Looney, R J (1986) Human leucocyte IgG Fc receptors Immunol Today 7, 264

Aminoff, D, Bell, W C, Fulton J and Ingebrigtsen, N (1976) Effect of sialidase on the viability of erythrocytes in circulation J Hematol 1, 419

Capel, P J A, Tamboer, W P M, De Waal, R M W, Jansen, J L J and Koene, R A P (1979) Passive enhancement of mouse skin allografts by alloantibodies is Fc dependent J Immunol 122, 421

Cooper P H, Mayer, P and Baggiolini, M (1984) Stimulation of phagocytosis in bone marrow-derived mouse macrophages by bacterial lipopolysaccharide correlation with biochemical and functional parameters J Immunol 133, 913

De Mulder, P H M, Wessels, J M C, Rosenbrand, D A, Smeulders J B J M, Wagener, D J Th and Haanen, C (1981) Monocyte purification with counterflow centrifugation monitored by continuous flow cytometry J Immunol Methods 47, 31

Jungi, T W (1985) A rapid and sensitive method allowing photometric determination of erythrophagocytosis by mononuclear phagocytes J Immunol Methods 82, 141

Kerbel, R S and Elliott B E (1983) Detection of Fc receptors Methods Enzymol 93, 113

Lemke, H, Krausse, R, Lorenzen, I and Havsteen, B (1985) Mycoplasma infection of cell lines can stimulate the expression of Fc receptors by binding of the carbohydrate moiety of antibodies Eur J Immunol 15, 442

Levin, M, Smith, C, Walters, M D S, Gascoine, P and Barratt, T M (1985) Steroid-responsive nephrotic syndrome a generalised disorder of membrane negative charge Lancet ii, 239

McCool, D, Birshtein, B K and Painter, R H (1985) Structural requirements of immunoglobulin G for binding to the Fc receptors of the human tumor cell lines U937, HL-60, ML-1, and K562 J Immunol 135, 1975

Michl, J, Pieczonka, M M, Unkeless, J C and Silverstein, S C (1979) Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages J Exp Med 150, 607

Naumann, H N (1964) The measurement of hemoglobin in plasma In F W Sunderman and F W Sunderman, Jr (Eds), Hemoglobin – Its precursors and metabolites (J P Lippincott Philadelphia, PA) p 40

Perussia, B, Dayton E T, Lazarus, R, Fanning, V and Trinchieri, G (1983) Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells J Exp Med 158, 1092

Rummage, J A and Leu, R W (1985) Photometric microassay for quantitation of macrophage Fc and C3b receptor function J Immunol Methods 77 155

Shah, V O, McCarley, D L and Weiner, R S (1984) Antibody-dependent cell-mediated cytotoxicity of cryopreserved human monocytes Cryobiology 21, 475

Tax, W J M, Willems, H W, Reekers, P P M, Capel, P J A and Koene, R A P (1983) Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells Nature 304 445

Tax, W J M, Hermes, F F M, Willems, H W, Capel, P J A and Koene, R A P (1984) Fc receptors for mouse IgG1 on human monocytes polymorphism and role in antibody-induced T cell proliferation J Immunol 133, 1185

Tax, W J M, Spits, H, Hermes, H F M, Willems, H W, Capel, P J A and Koene, R A P (1985) Polymorphism of human Fc receptors for mouse IgG1 and IgG2b Transpl Proc 17, 794

Unkeless, J C, Fleit, H and Mellman, I S (1981) Structural aspects and heterogeneity of immunoglobulin Fc receptors Adv Immunol 31, 247

Wessels, J M C and Veerkamp, J H (1973) Some aspects of the osmotic lysis of erythrocytes III Comparison of glycerol permeability and lipid composition of red blood cell membranes from eight mammalian species Biochim Biophys Acta 291, 190

Woof, J M, Partridge, L J, Jeffers, R and Burton, D R (1986) Localisation of the monocyte-binding region on human immunoglobulin G Mol Immunol 23, 319

CHAPTER 3

CHARACTERIZATION OF TWO Fc RECEPTORS FOR MOUSE IMMUNOGLOBULINS ON HUMAN MONOCYTES AND CELL LINES

Jan G.J. van de Winkel, Wil J.M. Tax, Mieke C.J. van Bruggen, Kees E.P. van Roozendaal, Riki W. Willems, Arjen Vlug, Peter J.A. Capel, and Robert A.P. Koene

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Characterization of Two Fc Receptors for Mouse Immunoglobulins on Human Monocytes and Cell Lines

J G J VAN DE WINKEL, W J M TAX, M C J VAN BRUGGEN, C E P VAN ROOZENDAAL H W WILLEMS, A VLUG P J A CAPEL & R A P KOENE

Department of Medicine Division of Nephrology and Department of Pathology St Radboud Hospital Nijmegen and Department of Immune Reagents Production Central Laboratory of The Netherlands Red Cross Blood Transfusion Service Amsterdam The Netherlands

Van de Winkel J G J Tax W J M Van Bruggen M C J Van Roozendaal C E P Willems H W Vlug A Capel P J A & Koene R A P Characterization of Two Fc Receptors for Mouse Immunoglobulins on Human Monocytes and Cell Lines *Scand J Immunol* **26**, 663–672 1987

We have previously reported a polymorphism in the mitogenic effect of murine (m) IgG1 anti CD3 monoclonal antibodies This polymorphism was genetically determined and could be attributed to polymorphism of the Fc receptor (FcR) for mIgG1 present on human monocytes We have now extended these studies by quantitating FcR expression on monocytes and cell lines by a recently developed EA rosette assay using the erythrocyte associated pseudoperoxidase activity The data show that the polymorphism of the monocyte FcR for mIgG1 is based on a quantitative rather than an absolute difference Furthermore this FcR is specific for mIgG1 and does not bind mIgG2a or mIgG2b nor surprisingly human IgG The expression of this FcR on cell lines correlates with their accessory function in IgG1 anti CD3 induced T cell proliferation mIgG2a can inhibit the rosetting of monocytes with erythrocytes sensitized with human IgG The FcR detected by this rosette technique can interact with all four human IgG subclasses but not with mIgG1 or mIgG2b The expression of this type of FcR on human cell lines correlates well with their ability to support mIgG2a anti CD3 induced mitogenesis These direct measurements of FcR expression support the concept that human monocytes have two independent FcR with affinity for mouse IgG one receptor specific for mIgG2a (which also binds human IgG) and a second specific for mIgG1

Jan G J van de Winkel Department of Medicine Division of Nephrology St Radboud Hospital Geert Grooteplein Zuid 8 6525 GA Nijmegen The Netherlands

Interaction of the Fc moiety of an antibody with an Fc receptor (FcR) can induce a great diversity of biological effects [23] The expression of FcR has been reported to be abnormal in a variety of human diseases [18] FcR may also be important for the immunotherapeutical use of murine monoclonal antibodies

Heterocytophilic interactions of murine immunoglobulins with human monocytes have been analysed in studies on the monocyte dependent induction of human T cell proliferation by antibodies against the CD3 antigen The mitogenic properties of anti CD3 antibodies were shown to be isotype dependent Antibodies of the mIgG2a subclass induced proliferation of T cells in essentially all human individuals although one defective family has been reported [4] mIgG1 anti CD3 antibodies, however were mitogenic in 70% of healthy Caucasian individuals but not in the remaining 30% [22] The induction of T cell proliferation by mIgG2b anti CD3 antibodies was observed in only a small minority (less than 10%) of normal individuals [21] We showed previously that the polymorphism of the mitogenic effect of mIgG1 anti CD3 antibodies is due to a polymorphism of

the FcR for mIgG1 on human monocytes [19] On the basis of both functional and biochemical studies, the presence of different FcR for human IgG has been suggested on human monocytes (reviewed by Anderson & Looney [1])

Cell lines that are thought to be representative of normal cells at a particular stage of maturation or differentiation have been extremely valuable for our present knowledge of FcR We compared the accessory function of cell lines (in antibody-induced T cell mitogenesis) and their FcR expression with a newly developed EA rosette assay [24] These variables were also studied on human monocytes The FcR on monocytes, mediating antibody-induced T cell proliferation, have previously been analysed by studying the inhibition of T cell mitogenesis by human sera or purified human IgG [10 15] We now report a detailed characterization by rosette inhibition studies of FcR on monocytes The results suggest the presence of two types of FcR on human monocytes with different affinities for homologous and heterologous IgG (of the various subclasses)

MATERIALS AND METHODS

Cell lines Human myeloid (KG1 HL 60) monocytic (U937) erythromyeloid (K562) B lymphoblastoid (Daudi) and T lymphoblastoid (Molt 4) cell lines were cultured in RPMI 1640 medium (Dutch modification containing both Hepes and sodium bicarbonate) supplemented with 10% heat inactivated fetal calf serum 2 mM glutamine 1 mM sodium pyruvate and gentamycin (50 μg/ml) This mixture will be referred to as complete medium

Monocytes Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats by centrifugation on a Percoll (Pharmacia Uppsala Sweden) gradient (s=1 072 g/ml) at 4°C Monocytes were purified from PBMC by counterflow centrifugation The final suspensions obtained by this procedure contained at least 90% monocytes (as determined by May–Grunwald–Giemsa and non specific esterase staining) Viability was greater than 98% as determined by trypan blue exclusion Because of the functional variability in monocytes from different individuals we considered it advantageous to have fixed and well defined batches of these cells The purified monocytes were therefore cryopreserved and used later Both the recovery and viability of cryopreserved monocytes were excellent (>70% and >95% respectively)

T lymphocytes Lymphocytes were purified from PBMC by elutriation T lymphocytes were isolated from this suspension by nylon wool filtration and rosetting with sheep erythrocytes treated with aminoethylisothiouronium bromide [3]

Ig preparations For the characterization of FcR on human monocytes three different murine monoclonal antibodies were used PA1 (IgG1 antibody against idiotypic determinant on B C LL cells produced by F Preyers Nijmegen) 3 1 3 1 (IgG2b anti mouse H 2 determinant) [14] and 2 2 1 (IgG2a anti mouse Ia determinant) [14] All antibodies were purified from ascites fluid by $(NH_4)_2SO_4$ precipitation followed by different chromatographic procedures [13] No residual antibodies of a contaminating subclass could be detected by double diffusion in agarose

Pure human IgG subclass proteins were isolated from the sera of patients with multiple myeloma After $(NH_4)_2SO_4$ precipitation and DEAE-Sephadex chromatography the isolated proteins were tested for impurities If necessary the preparations were further purified by affinity chromatography on Sepharose-protein A (IgG3) or Sepharose anti IgG subclass [8] The obtained proteins were checked for impurities and IgG subclass content in immunoelectrophoresis and radial immunodiffusion

Protein concentrations were determined spectrophotometrically at 280 nm with an extinction coefficient $E|_m$ of 14 3 for IgG

Binding of aggregated murine IgG Affinity purified mouse monoclonal antibodies at 30 mg/ml phosphate buffered saline (PBS) of the subclasses IgG2b IgG1 and IgG2a were aggregated by heating at 63°C for 2 18 and 25 min respectively Ten micrograms of each antibody preparation were incubated at 4°C with 10^6 monocytes Binding of aggregates was subsequently assayed by indirect immunofluorescence with fluorescein conjugated F(ab')$_2$ fraction of sheep anti mouse IgG antibody Cappel Malvern Pa USA) Fluorescence was determined with an Ortho 50 H flow cytometer In each sample the fluorescence intensity from 10 000 cells was measured

Sensitization of human erythrocytes A 0 5% suspension of human erythrocytes (HRBC) in PBS was incubated for 30 min at 37°C with various dilutions of antibody For mIgG1 EA rosetting we used ascites of an IgG1 monoclonal antibody against glycophorin A (a generous gift from M Bos CLB Amsterdam, The Netherlands) Human IgG EA rosetting was performed with Rhesus D positive HRBC and a commercially available polyclonal antiserum against Rhesus D (Merz & Dade Dudingen Switzerland) HRBC incubated with PBS alone served as controls for non-specific binding After incubation sensitized (and as a control unsensitized) HRBC were washed resuspended in RPMI 1640 containing 5% FCS and used within 30 min

Microscopic EA rosette assay Two hundred microlitres of a monocyte suspension (2×10^6 cells/ml in RPMI 1640 with 5% FCS) and 50 μl of sensitized HRBC suspension were mixed centrifuged (4 min at 20 g) and incubated for 1 h at 4°C Monocytes with at least three bound HRBC were scored as rosettes

Photometric EA rosette assay FcR expression can be quantitated with high sensitivity by using the erythrocyte associated pseudoperoxidase activity which we recently described in detail [24] Briefly monolayers of either monocytes or cell lines were prepared in 96 well flat bottomed microtitre plates (Costar Cambridge Mass USA) Sensitized HRBC were

dispensed into the wells and incubated for 1 h at 4°C, after which the plates were washed in PBS. The level of EA rosetting was quantitated by addition of a freshly prepared peroxidase substrate solution: 0·8 mg/ml 5′ aminosalicylic acid, dissolved in 50 mM phosphate buffer, pH 6·0, containing 0·072% v/v H_2O_2 to each well. After 30 min the extinction was measured at 450 nm in a Titertek® Multiskan.

To correct for variation between experiments in background absorbance values, a binding index (BI) was calculated for each experiment as follows:

$$BI=[1-(E450/\Sigma A450)]\times 100$$

where E450=extinction at 450 nm in wells to which HRBC *without* sensitizing antibody were added, ΣA450=extinction at 450 nm in wells to which *sensitized* HRBC were added. Duplicate samples were run for each dilution of antiserum used for sensitization of HRBC and the observed variation in BI values was small (usually less than 10%).

Rosette inhibition studies. Monocytes were dispensed into individual wells of microtitre plates and after adherence and washing away the nonadherent cells, 50 μl of inhibitor (or, as a control, RPMI 1640 alone) were added. The affinity-purified proteins, whose inhibitory effects were to be tested, were depleted of aggregates by ultracentrifugation (1 h at 100 000 *g*, 4°C) and rapidly snap frozen in small aliquots in liquid nitrogen. These proteins were stored at −20°C and thawed once just before use in the inhibition experiments. After incubation for 15 min at 4°C, 50 μl of a 1% suspension of sensitized HRBC (or as a control, unsensitized HRBC) in complete medium were added to each well and incubated for 45 min at 4°C. The level of FcR expression was quantitated by the photometric EA rosette assay. In all experiments the optimal level of HRBC sensitization was determined separately for each batch of monocytes. The inhibition studies were performed at a level of HRBC sensitization that only just gave a maximal BI in the photometric assay (edge of the binding plateau, see Results). The results are expressed as the percentage of inhibition, calculated as:

$$\left[1-\frac{EA450(+\text{inhibitor})-L450(+\text{inhibitor})}{EA450(\text{ inhibitor})-L450(-\text{inhibitor})}\right]\times 100$$

Lymphocyte stimulation with anti-CD3. The anti-CD3 antibodies used in the present study were WT32 (IgG2a) [20] and monoclonal antibody UCHT1 (IgG1), a kind gift from Dr Peter Beverley (London, UK) [3]. T cell proliferation in PBMC suspensions induced by anti-CD3 monoclonal antibodies was measured as described previously [22]. Briefly, mononuclear cells in complete medium were incubated for 72 h at 37°C with monoclonal antibody in U-bottomed wells of microtitre plates. The incorporation of [^{3}H]thymidine (added 18 h before the end of the incubation) was measured by liquid scintillation counting.

The accessory function of purified monocytes in the anti-CD3-induced mitogenesis of T cells was studied by incubating 2×10^4 monocytes, 1×10^5 purified T cells, and anti-CD3 antibody for 72 h at 37°C in U-bottomed microtitre wells. [^{3}H]thymidine uptake was assayed as described above.

When cell lines were used in T-cell proliferation experiments, these were first treated with mitomycin C. The cells were therefore incubated at $2·5\times10^6$ cells/ml for 60 min at 37°C with mitomycin C (100 μg/ml) (Sigma, St Louis, Mo., USA). The cells were washed three times with PBS, once with complete medium, and resuspended in complete medium. 1×10^5 mitomycin C-treated cells, 1×10^5 T cells, and anti-CD3 antibody were cultured for 72 h at 37°C, after which [^{3}H]thymidine incorporation was assayed.

RESULTS

Binding of aggregated murine IgG to monocytes

We characterized monocytes from over 100 human individuals for their accessory function in anti-CD3-induced T cell proliferation. Monocytes from all the tested individuals supported the proliferation of T cells by mIgG2a anti-CD3 antibodies. About 30% of all tested (Caucasian) individuals (low-responder individuals, LR) possessed monocytes that did not support T cell proliferation induced by mIgG1 anti-CD3 anti-

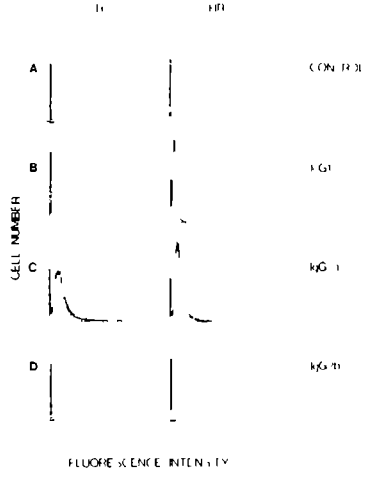

FIG. 1. Binding of aggregated antibodies of different isotypes to monocytes from an LR and an HR individual measured by indirect immunofluorescence. Histograms represent background staining (A) and binding of aggregated antibodies of the IgG1 (B, PA 1), IgG2a (C, 2-2-1), and IgG2b (D, 3-1-3-1) subclasses. The results are representative of similar experiments performed on monocytes from five LR and six HR individuals.

bodies (or only to a very limited extent at high antibody concentrations) Monocytes from the other donors support the induction of T cell proliferation by mIgG1 antibodies over a wide range of antibody concentrations (high-responder individuals HR) Monocytes did not support the proliferation induced by mIgG2b anti-CD3 antibodies The few exceptions that do respond to this subclass [21] were not included in our cell panel Aggregrated mIgG2a bound to monocytes from individuals of both the HR and LR phenotype (Fig 1) This is consistent with the accessory function of both monocyte batches in mIgG2a-induced T cell mitogenesis In contrast, mIgG2b aggregates did not bind, which correlates with the failure of mIgG2b anti-CD3 antibodies to induce T cell proliferation in these individuals HR monocytes bound significantly more mIgG1 aggregates than LR monocytes in all donors tested The fluorescence intensity obtained by the binding of mIgG1 aggregates to HR monocytes was low, and this assay is too insensitive for detailed characterization of this type of FcR on human cells FcR were therefore studied in other types of FcR assays

Differential binding of EA-mIgG1 by HR and LR monocytes

We have previously reported [19] that FcR for mIgG1 can be detected on monocytes from HR individuals by an EA rosette technique using HRBC sensitized with a monoclonal IgG1 antibody directed against glycophorin A The Fc dependency of EA rosetting was demonstrated by showing that HRBC sensitized with F(ab')$_2$ fragments of anti-glycophorin A antibody (at high concentrations) did not form EA rosettes, either counted visually or measured by the photometric assay [24] When very high concentrations of sensitizing antibody are applied, a low degree of rosetting is also seen with LR monocytes when rosettes are scored visually or by the photometric assay (Fig 2) At lower concentrations of anti-glycophorin antibody, rosettes are only formed with HR monocytes The apparent absence of FcR expression on LR monocytes as suggested from the immunofluorescence results with aggregated mIgG1 (Fig 1) is caused by the low sensitivity of this assay Both the immunofluorescence assay and the EA rosette assay, however, clearly discriminated between HR and LR individuals In the photometric EA rosette assay we regularly used 4×10^5 monocytes per well When lower cell concentrations were used, the plateau of BI, decreased in parallel (not shown) The dilution factor of antibody/antiserum at which half maximal EA rosetting is observed, the BI 50% value (BI_{50}) was found to be relatively constant (regardless of the number of cells per well) for a particular batch of monocytes (or cell line) Significant differences exist between HR and LR

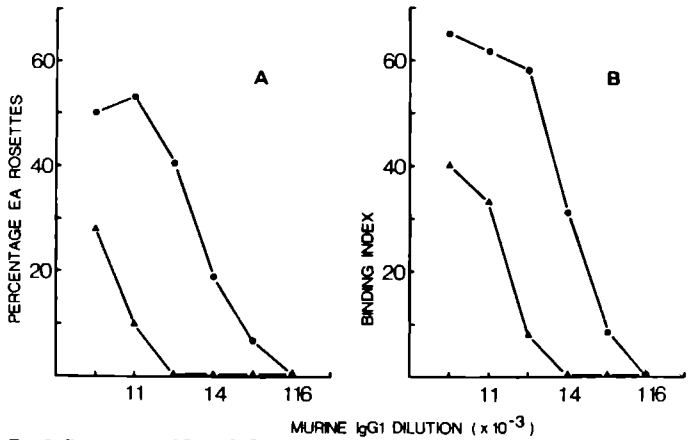

FIG 2 Comparison of EA-mIgG1 rosette formation by monocytes from an LR (▲) and an HR (●) individual by microscopic (A) and photometric (B) methods Each point in the figure represents the mean of duplicate determinations

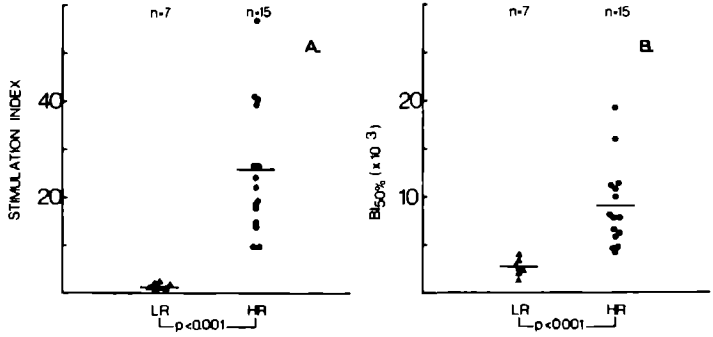

FIG 3 Comparison of HR and LR individuals in mIgG1 anti CD3 induced T cell proliferation and in a direct assay for mIgG1 FcR Accessory function (shown in A) was tested by incubating PBMC and monoclonal antibody UCHT1 (1 50 000 dilution of ascites) for 72 h at 37°C Stimulation indices were calculated by dividing cpm of incorporated [^{3}H]thymidine in the presence of UCHT1 by background cpm obtained in the absence of antibody BI_{50} values (presented in B) were determined by the photometric EA rosette assay Each point in the figure represents data from one individual the bars indicate means for the various groups Statistical analysis Wilcoxon two sample test

monocytes in both T cell mitogenesis and photometric EA rosette assays (Fig 3) In each individual photometric rosetting experiment, BI_{50} values observed for HR monocytes were found to be higher than the corresponding values for LR monocytes Furthermore, when using identical numbers of monocytes in the rosette assay, HR monocytes had a higher BI plateau than LR monocytes These results suggest a relative rather than an absolute difference in FcR expression for mIgG1 between monocytes from HR and LR donors

EA rosette inhibition studies

In order to characterize the FcR in more detail we performed inhibition studies with monomeric immunoglobulins In all experiments indicator erythrocytes were used sensitized with antibody concentrations giving optimal signals in the EA rosette assays FcR for mIgG1 were assayed by using indicator erythrocytes sensitized with a monoclonal anti-glycophorin A antibody By using Rhesus D positive HRBC sensitized with a human alloserum against Rhesus D we could detect FcR for human IgG with high sensitivity Haemagglutination assays indicated that in the latter case the indicator HRBC were coated mainly with human IgG1 and IgG3 (results not shown) FcR on monocytes detected by using EA-mIgG1 were markedly inhibited only by mIgG1 but not by mIgG2a or mIgG2b whereas the FcR for human IgG was strongly inhibited by mIgG2a but not at all by mIgG1 or mIgG2b (Fig 4) All human IgG subclasses were inhibitory for rosette formation of human monocytes with EA–human IgG (Fig 5) None of the human IgG subclasses gave significant inhibition of the mIgG1 rosetting Human immunoglobulins of

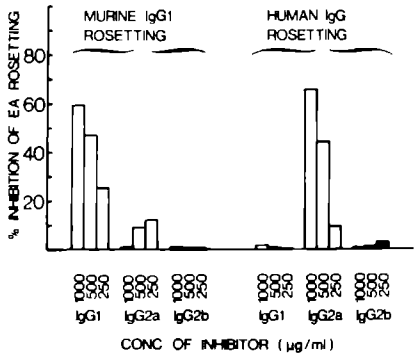

FIG 4 Inhibition of rosette formation between sensitized HRBC and human monocytes by monomeric murine immunoglobulins of different isotypes The results shown are the means of two experiments

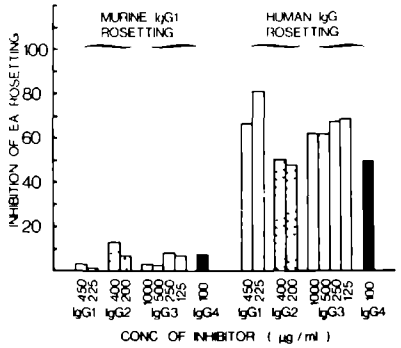

FIG 5 Inhibition of rosette formation between sensitized HRBC and human monocytes by monomeric human immunoglobulins of different subclasses The results shown are the means of three experiments

the classes IgM and IgA were ineffective as inhibitor in both types of EA rosetting with human monocytes (results not shown) Heterologous rabbit IgG has frequently been used to study FcR on human cells The inhibitory effects of rabbit and human sera (as a source of IgG) on both types of EA rosetting were tested Both sera inhibited the rosetting with human IgG-sensitized HRBC (80% inhibition at 2 5% serum), whereas mIgG1 EA rosetting remained unaffected (even at 10% serum, results not shown) All the data from the inhibition studies support the concept that different types of FcR are assayed by the two EA rosette assays

Correlation of FcR expression on human cell lines with accessory function in anti-CD3-induced T cell proliferation

When studying the accessory function of human cell lines in anti-CD3-induced T cell proliferation we observed a high degree of variation in stimulation indices between individual experiments Variation in FcR-mediated functions of cell lines between individual experiments is well known, and is probably related to changes in the number of FcR expressed during the various phases of the cell cycle [6] Despite these quantitative differences, however, the general pattern of accessory activity shown by the cell lines remained the same Because of this variability, both functional (T cell mitogenesis) and direct FcR assays were performed on the same day for each cell line In Table I the accessory function of different cell lines in mIgG1 anti-CD3-induced T cell proliferation is compared with EA rosetting All cell lines, with the exception of Molt-4, display rosetting with HRBC sensitized by the mIgG1 antibody The extent of accessory function correlates well with results from the rosette assay These results suggest that the FcR mediating the stimulation of T cells by mIgG1 anti-CD3 antibodies is identical to the one assayed by the photometric EA rosette assay Remarkably, U937 has a high stimulation index in the mitogenesis assay relative to its FcR expression

When FcR expression for human IgG was studied on cell lines (Table II), we observed a different pattern for several lines than was found for mIgG1 K562, for example, interacts well with mIgG1-sensitized HRBC but poorly with HRBC sensitized with human IgG The expression of FcR for human IgG as measured by EA rosetting is in accordance with accessory function in mIgG2a-induced T cell mitogenesis (Table II) This indicates that mIgG2a can interact with the FcR, which is assayed by the rosette assay using EA–human IgG, as was also sug-

TABLE I Correlation of FcR expression for murine IgG1 on human cell lines with accessory function in IgG1 anti-CD3-induced T cell proliferation

Accessory cells*	BI_{50}†	Stimulation index UCHT1 (IgG1)‡
Molt-4	0	1 0¶
Daudi	5300	7 9
HL-60	9600	14 4
KG1	11,200	14 2
U937	15,500	54 0
K562	56,000	53 8

r_s§ = 0 89
P < 0 02

*Accessory function was tested by incubating mitomycin C-treated accessory cells, T cells, and monoclonal antibody UCHT1 (1 50,000 dilution of ascites) for 72 h at 37°C

†BI_{50} values were determined by the photometric EA rosette assay

‡Stimulation indices were calculated as in legend to Fig 3

§ The relationship between the BI_{50} values and the observed stimulation indices was assessed using the Spearman rank correlation method

¶Results are representative for three experiments

TABLE II Correlation of FcR expression for human IgG on human cell lines with accessory function in IgG2a anti CD3 induced T cell proliferation

Accessory cells*	BI_{50} †	Stimulation index WT32 (IgG2a)‡
Molt 4	0	1 1¶
K562	2 5	2 1
Daudi	6 0	3 9
KG1	341	5 1
HL 60	427	15 8
U937	580	45 3

$r_S = 1\ 00$
$P < 0\ 001$

*Accessory function was tested by incubating mitomycin C treated accessory cells T cells and monoclonal antibody WT32 (50 ng/ml) for 72 h at 37°C

†BI_{50} values were determined by the photometric EA rosette assay

‡Stimulation indices were calculated as in legend to Fig 3

§For assessment of the relation between BI_{50}-values and the observed stimulation indices the Spearman rank correlation method was used

¶Results are representative for three experiments

gested from the inhibition experiments (Fig 4) Again, U937 displays high accessory function relative to its FcR expression

DISCUSSION

Previously we have analysed the interaction of human monocytes with mouse IgG in T cell proliferation studies and observed that mIgG2a anti CD3 antibodies were mitogenic with all donors and that mIgG1 and mIgG2b antibodies could induce T cell proliferation in only part of the human population [19 21 22] The polymorphism of the mitogenic effect of mIgG1 anti-CD3 antibodies was found to be due to polymorphism of the FcR for mIgG1 present on human monocytes [19] The FcR on human cells, mediating interactions with mIgG1 antibodies, are very difficult to study in direct assays Monomeric mIgG1 does not bind to a reasonable extent, and when using sheep erythrocytes sensitized with mIgG1 antibodies, no rosette formation could be observed [19] In the present study both immunofluorescence with aggregates of mIg, and EA rosetting (using HRBC) were used to detect FcR for different mIgG subclasses on human monocytes

Immunofluorescence with aggregates can be used as a direct assay for the presence of FcR, as illustrated in Fig 1, although fluorescence intensity is rather low Furthermore binding studies with aggregates tend to be poorly reproducible because of variations in the size and stability of aggregates in purity, and in the degree of monomer contamination Therefore we preferred to quantitate FcR by means of rosette assays The number of erythrocytes bound can be quantified by means of the pseudoperoxidase activity present in these cells [24]

Monocytes from HR individuals gave a higher degree of rosette formation at all dilutions of sensitizing antibody than LR monocytes FcR for mIgG1 could be demonstrated on LR monocytes only when exceedingly high concentrations of sensitizing antibody were used To our knowledge, this is the first direct evidence for relative rather than absolute differences in FcR expression of mIgG1 on monocytes from HR and LR individuals On the basis of mitogenesis experiments, Clement *et al* [5] have already suggested the presence of relative rather than absolute defects' in LR monocytes These investigators observed that monocytes from LR individuals could support antibody-induced T cell proliferation only at very high concentrations of mIgG1 anti-CD3 antibodies in contrast to HR monocytes, which also supported T cell proliferation at much lower concentrations Their results, however, can be explained by the presence of FcR for mIgG1 on LR monocytes, but another possibility is that coating of anti CD3 antibodies to the wells of the microtitre plates caused sufficient cross-linking to induce T cell proliferation Other authors have observed that anti CD3 antibodies on solid substrates (e g bound to Sepharose beads [19], or to goat anti mouse IgG coated microtitre plates [4] or coated directly to the wells of microtitre plates [7]) can induce T cell proliferation in the absence of monocytes

The data obtained with EA rosetting suggest that either far less FcR are present, or that the affinity of the FcR on LR monocytes for mIgG1 is much lower than on HR monocytes The second possibility is supported by studies of Looney *et al* [16], who described a monoclonal antibody (IV 3) with specificity for the FcR for mIgG1, which binds to both HR and LR monocytes with similar intensity (as judged to microfluorometry) Using this antibody, inhibition

studies in our own laboratory showed strong inhibition (>80%) of rosetting with EA-mIgG1 and no inhibition of hIgG EA rosettes, thus confirming the specificity of monoclonal antibody IV 3 for the FcR for mIgG1 (results not shown) Anderson *et al* [2] furthermore observed differences in isoelectric focusing patterns of purified FcR for mIgG1 from HR and LR monocytes

We found evidence from rosette inhibition studies for two types of FcR on human monocytes The first type can interact well with mIgG1-sensitized HRBC and can be inhibited by mIgG1 antibodies and not by mIgG2a or mIgG2b antibodies, human IgM, IgA or IgG or rabbit serum The second type of FcR interacts well with human IgG sensitized indicator erythrocytes This FcR can be inhibited by mIgG2a antibody and not by murine antibodies of the IgG1 or IgG2b isotypes Furthermore all human IgG subclasses as well as rabbit serum inhibited this receptor In this assay we used high concentrations of IgG subclasses to demonstrate clearly the difference in human IgG binding by the two types of receptors With this concentration range it is clear that the human IgG FcR can bind all four subclasses Binding affinity for the various subclasses however, may be different The finding that neither IgM nor IgA interacted with the two types of FcR on human monocytes emphasizes the importance of the Fc portion of IgG in the interaction with these FcR This was further substantiated by our finding that purified Fc fragments exhibited similar inhibition characteristics to whole human IgG (results not shown) Previously, other authors have described the inhibitory effects of human serum or human IgG on either anti-CD3-induced T cell proliferation [10, 15] or anti-CD3-induced interleukin 2 (IL-2) release from Jurkat cells [11] These studies indicate that only the mIgG2a-induced effects are inhibited by human IgG, and that the mIgG1-induced effects are not affected Furthermore, all four human IgG subclasses were found to inhibit the mIgG2a-induced human T cell mitogenesis [15]

Ideally we should have assayed the FcR on human cells mediating interactions with mIgG2a, by using erythrocytes sensitized with mIgG2a antibodies Such antibodies, however, were not available at the time when we performed the studies described above Recently we obtained mIgG2a switch variants of the (mIgG1) monoclonal anti-glycophorin A antibody used to assay the FcR for murine IgG1 Preliminary results show that the patterns of FcR expression on human cell lines obtained by using either human IgG- or mIgG2a-sensitized erythrocytes are similar, which substantiates our findings that both types of antibodies interact with the same receptor structure on human cells

Cell lines can be used as accessory cells in anti-CD3-induced T cell mitogenesis When the induction of T cell mitogenesis by anti-CD3 antibodies of the mIgG1 and mIgG2a subclasses was studied, different patterns emerged The expression of FcR for mIgG1 on cell lines correlated well with accessory function in mIgG1-induced mitogenesis The expression of FcR for human IgG correlated with the accessory function in mIgG2a-induced T cell proliferation of cell lines By using cell lines with variable expression of FcR for human IgG, we could positively identify this receptor as the one mediating interactions with mIgG2a antibodies in anti-CD3-induced T cell mitogenesis This is consistent with the data obtained in the rosette inhibition studies, where we found that mIgG2a inhibits the FcR for human IgG

A high level of rosette formation between U937 and HL-60 cells and EA-human IgG was observed, whereas K562 demonstrated little rosette formation with these indicator cells These results indicate that the FcR for human IgG on the U937 and HL-60 cell lines differ from those of the relatively undifferentiated K562 cell line These data correspond to the findings of other investigators, who have shown that K562 cells possess low affinity FcR, and U937 and HL-60 cells high affinity FcR specific for the Fc fragment of human IgG [17] The myeloid cell line KG1 appears to possess FcR for both mIgG1 and human IgG, which has not been reported before With heavily sensitized HRBC we could detect FcR for mIgG1 on several cell lines This type of FcR was previously reported to be absent on some of these lines (e g on U937 [12]) These results accentuate the sensitivity of our indicator system, and furthermore illustrate that the detection of FcR is strictly dependent on the type of assay and on the conditions applied

Only some cell lines were previously studied in functional assays Looney *et al* [16] studied the accessory function of Daudi and U937 cells in anti-CD3-induced T cell proliferation and observed that U937 supported the mitogenesis

induced by both mIgG1 and mIgG2a antibodies, whereas Daudi supported the mitogenesis by mIgG1 antibodies only Other investigators studied the induction of IL-2 release from Jurkat cells by murine anti-CD3 antibodies of different subclasses K562 could only support IL-2 release induced by mIgG1 anti-CD3 U937 supported IL-2 release by both mIgG1 and mIgG2a antibodies[11] Such results, which are in agreement with our data, indicate that functional assays may be used as indirect FcR assays

The interpretation of functional assays, however, is complicated for reasons such as the production of immunomodulatory factors, which varies between different cells (e g production of prostaglandin E [9]), and might significantly affect the results

In conclusion, the results of our studies support the concept that different human FcR are involved in the binding of murine IgG1 and IgG2a Further biochemical and functional studies will be necessary to characterize these receptors in more detail.

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REFERENCES

1 Anderson, C L & Looney, R J Human leucocyte IgG Fc receptors *Immunol Today* **7**, 264, 1986
2 Anderson, C L Rvan D H , Looney, R J & Leary P C Structural polymorphism of the human monocyte 40 kilodalton Fc receptor for IgG *J Immunol* **138**, 2254 1987
3 Beverley, P C L & Callard, R E Distinctive functional characteristics of human 'T lymphocytes defined by E rosetting or a monoclonal anti T cell antibody *Eur J Immunol* **11**, 329 1981
4 Ceuppens J L , Bloemmen, F J & Van Wauwe, J P T cell unresponsiveness to the mitogenic activity of OKT3 antibody results from a deficiency of monocyte Fc receptors for murine IgG2a and inability to crosslink the T3-T1 complex *J Immunol* **135**, 3882, 1985
5 Clement, L T , Tilden, A B & Dunlap, N E Analysis of the monocyte Fc receptors and antibody-mediated cellular interactions required for the induction of T cell proliferation by anti-T3 antibodies *J Immunol* **135**, 165 1985
6 Gandour, D M & Walker, W S Macrophage cell cycling influence on Fc receptors and antibody-dependent phagocytosis *J Immunol* **130**, 1108, 1983
7 Geppert, T D & Lipsky, P E Accessory cell independent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3 *J Immunol* **138**, 1660 1987
8 Goosen, P C M Isolation of subclasses of human IgG with affinity chromatography *J Immunol Methods* **37**, 89, 1980
9 Kurland, J I , Pelus, L M Ralph, P , Bockman, R S & Moore, M A S Induction of prostaglandin E synthesis in normal and neoplastic macrophages role of colony stimulating factor(s) distinct from effects on myeloid progenitor cell proliferation *Proc Natl Acad Sci USA* **76**, 2326, 1979
10 Landegren, U , Andersson, J & Wigzell, H Mechanism of T lymphocyte activation by OKT3 antibodies—a general model for T cell induction *Eur J Immunol* **14**, 325, 1984
11 Landegren, U , Andersson, J & Wigzell, H Analysis of human T lymphocyte activation in a T cell tumor model system *Eur J Immunol* **15**, 308, 1985
12 Lemke, H , Krausse, R , Lorenzen, I & Havsteen, B Mycoplasma infection of cell lines can stimulate the expression of Fc receptors by binding of the carbohydrate moiety of antibodies *Eur J Immunol* **15**, 442, 1985
13 Lems, S P M , Rijke Schilder, I P M , Capel, P J A & Koene R A P Similar activities of IgG1 and IgG2 alloantibodies in enhancement of mouse skin grafts and in opsonization *Transplantation* **37**, 291, 1984
14 Lems, S P M , Tax, W J M , Capel P J A & Koene, R A P Immune regulation by monoclonal antibodies failure to enhance mouse skin allografts *J Immunol* **131**, 86, 1983
15 Looney, R J & Abraham, G N The Fc portion of intact IgG blocks stimulation of human PBMC by anti-T3 *J Immunol* **133**, 154, 1984
16 Looney, R J , Abraham, G N & Anderson, C L Human monocytes and U937 cells bear two distinct Fc receptors for IgG *J Immunol* **136**, 1641, 1986
17 McCool, D , Birshtein, B K & Painter, R H Structural requirements of immunoglobulin G for binding to the Fc receptors of the human tumor cell lines U937, HL-60, ML-1 and K562 *J Immunol* **135**, 1975, 1985
18 Rhodes, J Altered expression of human monocyte Fc receptors in malignant disease *Nature* **265**, 253, 1977
19 Tax, W J M , Hermes, F F M , Willems, H W , Capel, P J A & Koene, R A P Fc receptors for mouse IgG1 on human monocytes polymorphism

and role in antibody induced T cell proliferation *J Immunol* **133,** 1185, 1984

20 Tax, W J M, Leeuwenberg, H F M, Willems, H W, Capel, P J A & Koene, R A P Monoclonal antibodies reactive with OKT3 antigen or OKT8 antigen Pp 721–722 in Bernard, A, Boumsell, L, Dausset, J, Milstein, C & Schlossman, S F (eds) *Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies* Springer Verlag, Berlin, 1984

21 Tax, W J M, Spits, H, Hermes, F F M, Willems, H W, Capel, P J A & Koene, R A P Polymorphism of human Fc receptors for mouse IgG1 and IgG2b *Transplant Proc* **17,** 794, 1985

22 Tax, W J M, Willems, H W, Reekers, P P M, Capel, P J A & Koene, R A P Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells *Nature* **304,** 445, 1983

23 Unkeless, J C, Fleit, H & Mellman, I S Structural aspects and heterogeneity of immunoglobulin Fc receptors *Adv Immunol* **31,** 247, 1981

24 Van de Winkel, J G J, Tax, W J M, Van Bruggen, M C J, Van Roozendaal, C E P, Willems, H W, Van Duijnhoven, J L P Capel, P J A & Koene, R A P A new photometric method for the quantitation of Fc receptors for murine IgG1 on human monocytes and cell lines *J Immunol Methods* **101,** 109, 1987

CHAPTER 4

A NEW RADIOMETRIC ASSAY FOR THE QUANTITATION OF SURFACE-BOUND IgG ON SENSITIZED ERYTHROCYTES

Jan G.J. van de Winkel, Wil J.M. Tax, Arie Groeneveld,
Wim P.M. Tamboer, Pieter H.M. de Mulder, and Peter J.A. Capel

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A new radiometric assay for the quantitation of surface-bound IgG on sensitized erythrocytes

J.G.J. Van de Winkel, W.J.M. Tax, A. Groeneveld [1], W.P.M. Tamboer, P.H.M. De Mulder [1] and P.J.A. Capel

Department of Medicine, Divisions of Nephrology and [1] Medical Oncology, University Hospital Nijmegen, Nijmegen, The Netherlands

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We have developed a sensitive, straightforward method for the quantitation of surface-bound IgG on sensitized erythrocytes. The assay is based on the consumption by sensitized cells of anti-IgG antiserum The remaining anti-IgG is quantitated in a second incubation by precipitation with ^{125}I-IgG in the presence of polyethylene glycol. Calibration curves for this assay were constructed using known amounts of unlabeled IgG. The method can be performed in microtitre plates and eliminates the use of purified anti-erythrocyte antibodies, or highly purified specific anti-IgG antisera. The results were completely consistent with those of immunofluorescence assays, but our method was much more sensitive, less than 500 molecules of IgG per cell being detected reproducibly. The technique is not laborious and takes much less time than previously described methods with similar sensitivity

As an example of the applicability of this test, the implications of ligand density for the detection by EA rosetting of Fc receptors on human monocytes are shown. The results suggest that a large variation exists in the affinity of the different types of Fc receptors for their ligands.

Key words Sensitized erythrocyte; Radiometric assay, Fc receptor, human; Antibody-coated erythrocyte rosetting

Introduction

Erythrocytes are widely used as indicator cells. Many erythrocyte membrane antigens are well characterized and many antisera of known specificity are readily available. Furthermore, the immobility of surface components in the HRBC membrane, which is relatively unique among eukaryotic cells (reviewed by Singer, 1974), is advantageous in many types of assay. Antibody-sensitized human erythrocytes are frequently used as targets in ADCC, and in the detection and quantitation of FcR (EA rosette assays). EA rosetting using antibody-sensitized HRBC can be very sensitive, and FcR have been described which can be detected using sensitized HRBC but not the sensitized erythrocytes from other species (e.g., the FcR for mIgG1 on human monocytes; Tax et al., 1984). An important aspect in the detection of FcR is the degree of erythrocyte sensitization and this can be very high on HRBC when suitable antibodies are used. The well-known dependence

Correspondence to J.G.J. Van de Winkel, Department of Medicine, Division of Nephrology, University Hospital Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, The Netherlands

Abbreviations ADCC, antibody-dependent cell-mediated cytotoxicity; B-CLL, chronic lymphocytic leukemia of B origin, BSA, bovine serum albumin, CD, cluster of differentiation, EA, antibody-coated erythrocyte, FcR, receptor(s) for the Fc fragment of Ig, h, human, HR, high responder, HRBC, human erythrocyte(s), IF, immunofluorescence, LR, low responder; m, murine, PBS, phosphate-buffered saline, PEG, polyethylene glycol 6000, RIA, radioimmunoassay

of rosette formation and ADCC on the degree of antibody sensitization (Shaw et al, 1979) emphasizes the need to study this variable in more detail

Antibody density on sensitized erythrocytes can be quantitated by several techniques When the anti-erythrocyte antibody is available in purified form (which is generally not the case), direct binding studies can be performed (Jungi and Barandum, 1985) Assays based on the use of ^{125}I-labeled anti-IgG have been described (Merry et al, 1982), but these were laborious and suffered from problems such as nonspecific binding of the anti-IgG to red cells (Rearden and Masouredis, 1980) Membrane-bound IgG can also be quantitated using ^{125}I-protein A (Langone et al, 1977), but this method suffers from disadvantages such as unknown and variable binding stoichiometry, and absence of affinity of protein A for certain subclasses of immunoglobulins

In this report we present a new, straightforward technique by which the degree of erythrocyte sensitization can be quantitated This method essentially measures the decrease in the capacity to precipitate ^{125}I-labeled IgG by an anti-IgG antiserum, which has previously been incubated with sensitized HRBC The assay can be performed in microtitre plates, and is much less time consuming than previously reported methods The method has been applied to quantitate the binding of both a monoclonal mouse antibody and a polyclonal human antiserum against well-characterized antigens on HRBC (glycophorin A, and rhesus D antigen, respectively) The mouse IgG1 antibody against glycophorin A has been used previously in an EA rosetting assay to demonstrate that a genetic polymorphism exists in the Fc receptor for mouse IgG1 on human monocytes (Tax et al, 1984) This polymorphism in Fc receptors for mouse IgG1 is in turn responsible for a polymorphism in the induction of T cell proliferation by mouse IgG1 anti-CD3 antibodies (Tax et al, 1983) and defines the so-called high-responder (HR) and low-responder (LR) phenotypes The quantitative data obtained with the radiometric assay are discussed in relation to the detection of the Fc receptor for human IgG, and the HR and LR Fc receptor for mouse IgG1

Materials and methods

Ig preparations

For the quantitation of mIgG1 antibody molecules on sensitized HRBC, we used a murine IgG1 monoclonal antibody for the construction of calibration curves This antibody, PA1 (directed against an idiotypic determinant on B-CLL cells, produced in our laboratory by F Preyers) was purified from ascites fluid by $(NH_4)_2SO_4$ precipitation, followed by different chromatographic procedures as described by Lems et al (1984) No residual antibodies of subclasses other than mIgG1 could be detected by double diffusion in agarose (i e, more than 99 9% pure)

The degree of HRBC sensitization by human antibodies was determined by using hIgG, purified from human serum by ammonium sulphate precipitation and DEAE-Sephadex (Pharmacia, Uppsala, Sweden) chromatography The final preparation of human IgG was checked for impurities and IgG content in immunoelectrophoresis and radial immunodiffusion In some experiments purified hIgG3 was used, isolated from the sera of patients with myelomas by ammonium sulphate precipitation and affinity chromatography The hIgG preparations (99 9% pure) were from the Department of Immune Reagents Production, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam

After isolation, all fractions were depleted of aggregates by ultracentrifugation (1 h at 100 000 × *g*, 4°C) and the protein concentrations determined by the method described by Bradford et al (1976) using bovine gamma globulin (Bio-Rad, Munich, F R G) as a standard The IgG preparations were diluted in PBS, containing 0 1% sodium azide and 0 1% BSA (Cohn fraction V, Sigma, St Louis, MO, U S A), and snap frozen in small aliquots in liquid nitrogen The proteins were stored at −20°C and thawed once, just before use in the radiometric experiments

Radioiodination

After ultracentrifugation a fraction of the purified Ig of both mIgG1 and hIgG was used for the preparation of radiolabeled antigen 50 μg of the antibodies were labeled with 0 3 mCi Na^{125}I (Ra-

diochemical Centre, Amersham, U K), using the chloramine-T method (Hunter, 1973), resulting in a specific activity of 3 μCi/μg ^{125}I-IgG Free iodine was separated from protein-bound label by Sephadex G-25 (Pharmacia, Uppsala, Sweden) chromatography Labeled proteins were diluted in PBS, containing 0 1% NaN_3 and 0 1% BSA, and stored at 4° C for up to 1 month

Sensitization of human erythrocytes

To obtain sensitized HRBC, a 0 5% suspension of the cells (in PBS containing 0 1% BSA) was incubated for 30 min at 37° C with various dilutions of antibody HRBC, sensitized with mIgG1, were obtained using ascites of an IgG1 monoclonal antibody against glycophorin A (a kind gift from M Bos, CLB, Amsterdam, The Netherlands) HRBC coated with hIgG antibody molecules were prepared using a human polyclonal antiserum against rhesus D (Merz & Dade, Dudingen, Switzerland) HRBC incubated with PBS (with 0 1% BSA) alone served as controls (unsensitized erythrocytes) in the radiometric assay. After incubation, sensitized HRBC were washed twice, resuspended and used immediately

The rhesus phenotypes of the HRBC were determined at the Blood Bank, Nijmegen, The Netherlands

Immunofluorescence

The binding of different Ig preparations to HRBC was estimated by using indirect immunofluorescence 100 μl of a 1% suspension of sensitized HRBC in IF buffer (PBS containing 1% BSA and 0 1% sodium azide) were incubated with an equal volume of (Fab fragments of) fluorochrome-labeled antisera, diluted 1 in 50 in IF buffer HRBC, sensitized with mIgG1, were incubated with goat anti-mouse Ig (H&L)-FITC (Cappel, Malvern, PA, U S A), and hIgG-sensitized HRBC with goat anti-human IgG (H&L)-FITC (Cappel, Malvern, PA, U S A) After a 30 min incubation at room temperature, the cells were washed two times, fixed with 1% paraformaldehyde and subsequently analyzed by an Ortho 50-H flow cytometer The fluorescence intensity from 10000 cells was determined, and the mean fluorescence intensity was calculated from the histograms

Radiometric assay

The erythrocyte-bound antibodies were quantitated by their ability to inhibit the binding of standardized amounts of ^{125}I-IgG by anti-IgG antisera The binding of ^{125}I-IgG was quantitated by precipitation of the immune complexes with 6% PEG, a concentration which did not affect uncomplexed antibodies

The optimal amount of anti-IgG to precipitate a fixed amount of ^{125}I-IgG was determined as follows A dilution series of either rabbit anti-human IgG (CLB, Amsterdam, The Netherlands), prepared by immunization of animals with Fc fragments of hIgG1 and hIgG2, sheep anti-human IgG3 (Nordic, Tilburg, The Netherlands) or goat anti-mouse IgG1 (Meloy, Springfield, VA, U S A) antisera was incubated with a fixed amount of radiolabeled human IgG, IgG3 or mouse IgG1, respectively (approx 10000 cpm/well) in a final volume of 150 μl in PBS, supplemented with 0 07% BSA, 0 1% sodium azide, and a final concentration of 6% PEG for 18 h at 4° C in round-bottomed microtitre plates (Costar, Cambridge, MA, U S A) Thereafter the plates were centrifugated (45 min at 1600 × g, 4° C), 75 μl of the supernatants were transferred to Micronic tubes (Flow, Irvine, U K) and radioactivity was counted in an LKB gamma counter The percentage precipitation of labeled IgG was calculated by comparison with the value obtained in the absence of antiserum In all cases this last value deviated less than 5% from the input, indicating no spontaneous precipitation of ^{125}I-IgG in the presence of 6% PEG The minimal amount of antiserum which still precipitated the maximal amount of ^{125}I-IgG was further used in the assays

A calibration curve was obtained by the incubation of a dilution series of known amounts of human or mouse IgG for 1 h at room temperature with the optimal amount of antiserum in a final volume of 100 μl After centrifugation (10 min at 600 × g, room temperature), 50 μl of this mixture was placed in fresh microtitre wells and incubated with a fixed amount of ^{125}I-IgG for 18 h at 4° C in an end-volume of 150 μl, with a final concentration of 6% PEG in PBS containing 0 07% BSA, 0 1% NaN_3, and further handled as described above

To determine the total amount of erythrocyte-

bound antibody, variable numbers of sensitized HRBC were incubated with the fixed amount of anti-IgG sera in the same way as described for the calibration curve Controls without HRBC and with unsensitized HRBC were included in each experiment In both types of control a maximal precipitation of labeled antigen was found in all experiments, indicating that none of the antisera bound nonspecifically to HRBC or to the wells of the microtitre plates After incubation, the microtitre plates were centrifuged for 10 min at 600 × *g* (room temperature) and 50 μl of the supernatants were incubated with ^{125}I-IgG in the presence of 6% PEG in a final volume of 150 μl for 18 h at 4°C, and further handled as described above, and the percentage inhibition of the precipitation of ^{125}I-IgG was calculated The quantity of HRBC-associated IgG could be calculated using the calibration curve, determined in the same experiment, and converted to molecules per cell, because the number of HRBC was known The molecular weight of IgG was assumed to be 160 000, and it can be calculated that 1 ng of IgG corresponds to approximately 3.8×10^9 molecules of IgG

All radiometric determinations were performed in triplicate and the means are shown in the figures All experiments were performed at least three times and representative experiments are shown

EA rosetting of human monocytes

Monocytes were purified from buffy coats as previously described (Van de Winkel et al, 1987) The HR and LR phenotype of monocytes from different donors was studied in mitogenesis assays as described by Tax et al (1983) The induction of human T cell proliferation by murine anti-CD3 antibodies UCHT1 (mIgG1, a kind gift from Dr Peter Beverley, London, U K), and as a positive control WT32 (mIgG2a, Tax et al, 1983) was studied with isolated monocytes as accessory cells

After isolation and characterization in mitogenesis assays, EA rosette formation between monocytes and HRBC sensitized with different types of antibody molecules was performed as previously described (Van de Winkel et al, 1987) Briefly, sensitized HRBC (prepared as described above) were dispensed into microtitre plates, containing monolayers of monocytes, and incubated for 1 h at 4°C The extent of EA rosetting was quantitated using the erythrocyte-associated pseudoperoxidase activity, measured photometrically at 450 nm A binding index was calculated for each experiment (to correct for variation in background absorbance values) as follows

$$[1 - (E_{450}/F_{A450})] \times 100 = \text{binding index}$$

where E_{450} is extinction at 450 nm in wells to which HRBC *without* sensitizing antibody were added, and E_{A450} is extinction at 450 nm in wells to which *sensitized* HRBC were added

The HRBC suspensions sensitized with different dilutions of antiserum were tested in duplicate and each experiment was performed at least three times

Results

Quantitation of IgG antibody molecules on sensitized HRBC

The principle of the radiometric assay used for quantitation of the degree of HRBC sensitization is illustrated in Fig 1 A titration curve was constructed for each antiserum, to estimate the appropriate working dilution (Fig 1*A*) Both unlabeled soluble IgG (calibration curve) and IgG bound to the surface of sensitized HRBC consumed anti-IgG antiserum, resulting in a diminished binding of ^{125}I-labeled IgG in the second incubation (Figs 1*B* and 1*C*) The amount of surface-bound IgG was determined by interpolation in the linear part of the calibration curve, at equivalent percentages of precipitated labeled IgG A percentage close to 50% of the maximal precipitation was chosen for this calculation

The number of sensitized HRBC used per assay varied with the degree of sensitization HRBC coated with optimal amounts of mIgG1 anti-glycophorin A antibody already caused a measurable signal in the assay when 2.5×10^4 cells were used, whereas 5×10^5 cells, sensitized with human antibodies against rhesus D, had to be used to observe detectable inhibition of the secondary precipitation reaction Unsensitized HRBC were included in each experiment, and were never found to bind

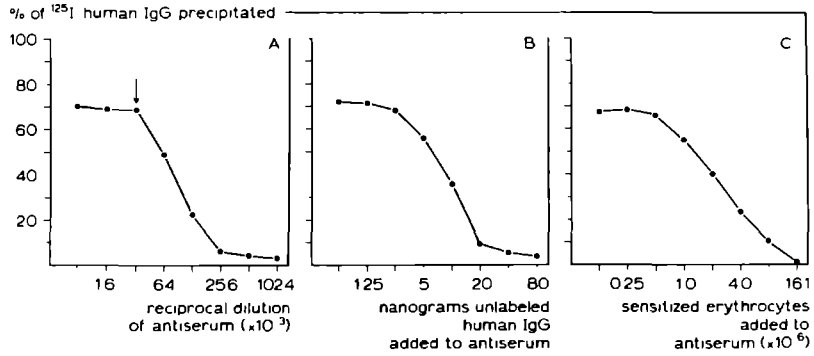

Fig 1 Radiometric analysis of the degree of HRBC sensitization by a human anti rhesus D antiserum *A* Titration curve for anti-IgG antiserum The amount of ^{125}I-hIgG which was precipitated (in the presence of PEG) by anti-hIgG is plotted against the serial dilution of the antiserum The arrow indicates the antiserum dilution used for *B* and *C* *B* Inhibition of the precipitation reaction by previous incubation of anti-hIgG antiserum with known amounts of soluble hIgG (calibration curve) *C* Inhibition of the precipitation reaction by previous incubation of anti hIgG antiserum with HRBC sensitized with human anti rhesus D antibody Rhesus phenotype of HRBC CCDee

anti-IgG antiserum The maximal number of HRBC used per assay was 5×10^7, at which concentration no agglutination was observed

Comparison of radiometric and fluorometric assays in the detection of cell surface-bound IgG

When the dilution of sensitizing anti-erythrocyte antibodies was varied the amount of cell-bound IgG was also regulated Fig 2 shows that a sigmoid curve was obtained when the amount of cell-bound mIgG1 (measured by the radiometric assay) was plotted against the concentration of sensitizing anti-glycophorin A monoclonal antibody A similar curve was obtained when the amount of cell-bound mIgG1 was evaluated by indirect immunofluorescence The radiometric assay, however, was much more sensitive When the amount of cell-bound murine IgG1 was lower than approximately 2×10^4 molecules/cell, immunofluorescence could no longer discriminate, whereas radiometry still did This effect was even more pronounced when HRBC were sensitized by a human anti-rhesus D antiserum (Fig 3) In this case, the level of sensitization was much lower than with mIgG1-sensitized cells, and immunofluorescence could not discriminate between the different degrees of coating, despite the fact that positive cells were identified by the cytofluorometer

Since a human alloantiserum was used for the detection of rhesus D antigens on HRBC, we were

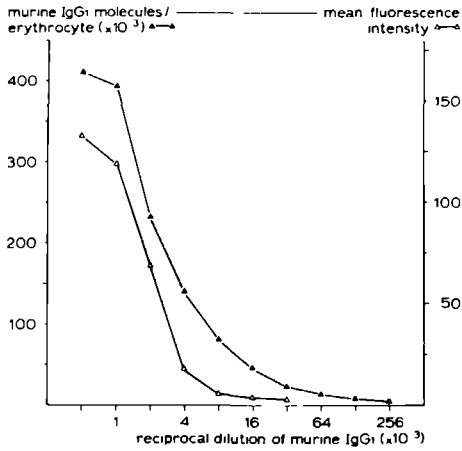

Fig 2 Sensitization of HRBC with different dilutions of murine IgG1 anti-glycophorin A monoclonal antibody, quantitated by the radiometric method (▲) and by indirect immunofluorescence (△)

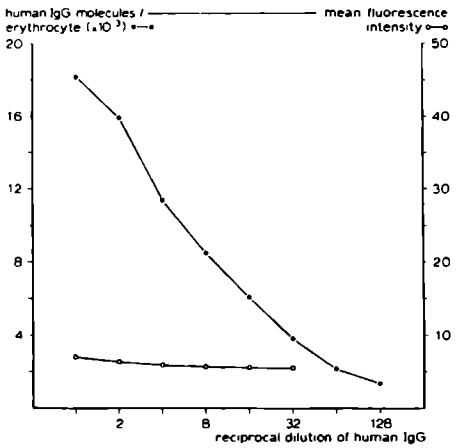

Fig. 3 Sensitization of rhesus D-positive HRBC (phenotype: CCDee) with different dilutions of human anti-rhesus D antiserum, quantitated by the radiometric assay (●) and by indirect immunofluorescence (○).

interested to know which of the human subclasses bound to the cells. Indirect haemagglutination assays indicated that human IgG1 and IgG3 were present (results not shown). The radiometric assay revealed that 60 ± 14% (mean ± SD, six individual experiments) of the sensitizing antibody were IgG3.

Reproducibility and sensitivity of the radiometric method

The maximal degree of HRBC sensitization was measured for HRBC from 14 different individuals (Table I). Anti-glycophorin A antibody sensitized HRBC to a much higher degree than anti-rhesus D antiserum. Variations between triplicates, as well as variations observed between different samples (of sensitized HRBC) from one donor, within each individual experiment were smaller than 5%. Furthermore, when the sensitization of HRBC from one batch was measured more than once, the variation between the individual experiments was less than 10% (for both types of antibody; results not shown). It can be observed that the anti-rhesus D antiserum gave a low degree of sensitization of rhesus D-negative HRBC. Anti-glycophorin A antibody sensitized rhesus D-positive and -negative HRBC to a comparable degree.

A few nanograms of IgG can be detected by the radiometric assay (Table II). The minimal

TABLE II

DETECTION LIMITS OF THE RADIOMETRIC ASSAY

Type of IgG	n [a]	Minimal detectable amount of unlabeled inhibitor [b] (ng/assay)	Minimal detectable degree of HRBC sensitization (molecules/HRBC)
Murine IgG1	15	2.7 ± 0.6 (1.8–4.2) [c]	160–250 [d]
Human IgG	12	3.5 ± 1.3 (1.5–6.4)	170–360

[a] Number of experiments performed

[b] Each value represents the lowest amount of unlabeled IgG causing more than 20% inhibition in an individual experiment

[c] Mean and standard deviation is given, and (in brackets) the range of values obtained

[d] Calculated for maximal number of erythrocytes/assay (5×10^7)

TABLE I

QUANTITATION OF IgG ANTIBODY MOLECULES ON OPTIMALLY SENSITIZED HUMAN ERYTHROCYTES

Sensitizing antibody	Erythrocytes	n [a]	Estimated number of IgG antibody molecules/erythrocyte ($\times 10^{-3}$)
Anti-glycophorin A monoclonal antibody (murine IgG1)	HRBC	16	419 ± 50 (344–493) [b]
Anti-rhesus D polyvalent antiserum (human IgG1 + IgG3) [c]	D-positive HRBC	10	19 ± 3 (17– 26) [d]
Anti-rhesus D polyvalent antiserum (human IgG1 + IgG3)	D-negative HRBC	4	2 ± 1 (2– 3)

[a] Number of experiments performed.

[b] Mean and standard deviation is given, and (in brackets) the range of values obtained in individual experiments

[c] Assessed by haemagglutination.

[d] Rhesus phenotypes of HRBC were ccDEe (two donors), CcDee (five donors), CcDEe (one donor), CCDee (two donors), and ccdee (four donors).

amount of molecules per cell that can be assayed was calculated by dividing the lowest detectable amount of IgG by the maximal number of HRBC per assay which can be used Table II shows that less than 500 molecules of IgG per cell could be easily assayed by the radiometric method

EA rosetting using HRBC sensitized by human IgG or murine IgG1

Antibody-coated erythrocytes (EA) can be used to detect and quantitate FcR on cells We used HRBC sensitized with human IgG or murine IgG1 to measure the expression of the FcR for human IgG, and the polymorphic FcR for mIgG1, respectively Monocytes of both the HR and the LR phenotype were used in these studies When the effect of ligand density was studied, striking differences were observed between human IgG-sensitized and mIgG1-sensitized HRBC (Fig 4) Approximately 2700 molecules of human IgG/HRBC produced half-maximal rosetting with human monocytes In contrast, approximately 85 000 molecules of mIgG1/HRBC were needed for half-maximal rosetting with HR monocytes In order to get half-maximal rosetting with LR monocytes and EA-mIgG1, approximately 165 000 molecules/cell were needed Apart from the fact that twice as many mIgG1 molecules/HRBC were needed to obtain half-maximal rosetting with LR monocytes, compared to HR monocytes, the maximal level of rosette formation was also lower for

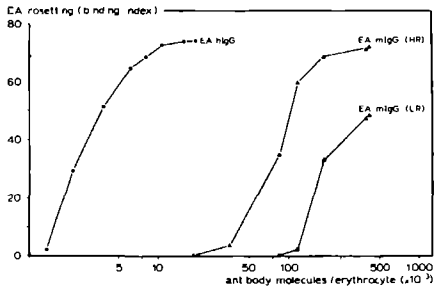

Fig 4 Effect of ligand density on the EA-rosette formation of human monocytes with EA hIgG (●) and EA mIgG1 Rosetting with EA-mIgG1 was investigated for monocytes from individuals of both the HR (▲) and LR (△) phenotype

LR monocytes No differences were observed between HR and LR monocytes with respect to human IgG EA rosette formation

Discussion

In this report we describe a very sensitive method for the quantitation of surface-bound IgG on sensitized erythrocytes The data from the radiometric assay correlated well with immunofluorescence measurements The radiometric assay was, however, considerably more sensitive A few hundred molecules of IgG/cell could be detected reproducibly

Other methods of a similarly high sensitivity have been described Dixon et al (1975) described a complement lysis inhibition assay for the quantitation of anti-platelet antibodies, which was modified by Kurlander et al (1978) to study antibody binding to HRBC This technique, however, was 'cumbersome and not suitable for routine use' (Dixon et al, 1975) Smith et al (1970) described a technique for the measurement of immunoglobulin bound to human lymphocytes, which was based on the binding of anti-IgG serum to cells, and quantitation of residual binding to ^{125}I-labeled Fab in the same tube Saturated ammonium sulphate was used to precipitate ^{125}I-Fab-antibody complexes in a one-step assay This procedure was very laborious and took at least 3 days Other assays based on the consumption of anti-Ig antisera by sensitized cells, and subsequent quantitation by radioimmunoassay were described by Rabellino et al (1971) for the quantitation of surface Ig on human lymphocytes, and by Jensenius and Williams (1974) for the quantitation of rat Ig in detergent lysates of cells All these methods are considerably more laborious and time consuming than our method and cannot be performed in microtitre plates

Using the radiometric assay we found an average of 19 000 molecules of anti-rhesus D antibody bound to each HRBC This value is in accordance with those obtained by other methods (e g, Kurlander et al, 1978, Jungi and Barandun, 1985) Rochna and Hughes-Jones (1965) observed differences in D antigen expression between various phenotypes, although a considerable overlap was

found for the more 'common' phenotypes Hughes-Jones et al (1971) studied D antigen expression on 'rare' phenotypes (e g , -D-), and observed much higher numbers of D antigen sites on these cells In this study we have used only HRBC of the more general phenotypes, and our data correspond very well with those reported by Rochna and Hughes-Jones (1965) Most authors have reported the degree of erythrocyte sensitization after subtraction of binding to rhesus D-negative HRBC Our finding that a small amount of anti-rhesus D antiserum binds to rhesus D-negative HRBC, is generally observed (and corrected for by subtraction of binding to rhesus D-negative HRBC, e g , Kurlander et al , 1978, Merry et al , 1982) This nonspecific binding may be related to the fact that an alloantiserum was used, which presumably was not completely devoid of other reactivities We feel that it is better to give total rather than corrected values for erythrocyte sensitization, because all sensitizing antibodies are important for interaction with FcR

Our estimate of the total amount of anti-glycophorin A antibody molecules bound per HRBC (approximately 419 000 molecules/ cell) is in agreement with the values reported by others Marchesi et al (1972) estimated that the total amount of glycophorin A molecules/HRBC was about 500 000 These data indicate that the radiometric assay can be used for quantitative measurements Furthermore, it appears that the anti-glycophorin A monoclonal antibody we used is special in that it can give an extremely high degree of sensitization, without causing agglutination of the HRBC Other anti-glycophorin A monoclonal antibodies tested by us, e g , E11B (Ochiai et al , 1983) were able to sensitize HRBC to about 200 000 molecules/cell but under such conditions agglutination was observed When this antibody was diluted to a concentration which no longer caused agglutination, only 10 000 molecules of IgG were found to be bound/HRBC, which was too low for detection of the mIgG1 FcR on human monocytes

Sensitized HRBC can be used to quantitate FcR on human monocytes (Van de Winkel et al , 1987) Our results suggest that large differences exist in the affinity of different types of FcR for their ligands Rhesus D-positive HRBC sensitized with human IgG at less than 3000 molecules/cell, yielded half-maximal rosetting with human monocytes This value is similar to the one found by Kurlander et al (1978) for the phagocytosis of anti-rhesus D-sensitized HRBC by monocytes At least 30 times more murine IgG1 molecules/ HRBC have to be present for half-maximal rosetting with monocytes from HR individuals Although variations in relative antigen mobility may underly such differences, surface components in the HRBC membrane tend to be immobile (Singer, 1974) The most likely explanation for these findings is that the affinity of the mIgG1 FcR on human monocytes for its ligand is very low LR monocytes need indicator cells with approximately twice as many mIgG1 molecules/HRBC for half-maximal rosetting, compared with HR monocytes The very high numbers of anti-erythrocyte antibody molecules/cell required for rosetting with mIgG1-coated indicator cells may also explain the inability of several authors to detect this type of FcR on human monocytes Using sheep erythrocytes sensitized with the mIgG1 monoclonal antibody MAS 014 (Sera-lab, Sussex, U K) we observed that up to 70 000 molecules/cell could be detected This degree of sensitization may be too low to promote sufficient interaction with the mIgG1 FcR to result in EA rosette formation since we were unable to detect the mIgG1 FcR using these sensitized sheep erythrocytes (Tax et al , 1984)

In conclusion, we describe a new, sensitive, and reproducible method for the detection of antibody molecules on sensitized erythrocytes The assay can be performed in microtitre plates, is not laborious, and the total procedure takes less than 20 h We have used this method to analyze the degree of sensitization of HRBC which is required for EA rosetting to occur Our results show that for rosetting a much higher number of murine IgG1 antibodies must be bound to the surface of HRBC compared to the amounts of human IgG required This method can also be used for the quantitation of antibody binding to other cells

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References

Bradford, M M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal Biochem 72, 248

Dixon, R, Rosse, W and Ebbert, L (1975) Quantitative determination of antibody in idiopathic thrombocytopenic purpura New Engl J Med 292, 230

Hughes-Jones, N C, Gardner, B and Lincoln, P J (1971) Observations of the number of available C, D, and F antigen sites on red cells Vox Sang 21, 210

Hunter, W M (1973) Radioimmunoassay In D M Weir (Ed), Handbook of Experimental Immunology, Blackwell, Oxford p 17 1

Jensenius, J C and Williams, A F (1974) Total immunoglobulin of rat thymocytes and thoracic duct lymphocytes Eur J Immunol 4, 98

Jungi, T W and Barandun, S (1985) Estimation of the degree of opsonization of homologous erythrocytes by IgG for intravenous and intramuscular use Vox Sang 49, 9

Kurlander, R J Rosse, W F and Logue, G L (1978) Quantitative influence of antibody and complement coating of red cells on monocyte-mediated cell lysis J Clin Invest 61, 1309

Langone, J J, Boyle, M D P and Borsos, T (1977) ^{125}I protein A applications to the quantitative determination of fluid phase and cell bound IgG J Immunol Methods 18, 281

Lems, S P M, Rijke-Schilder, T P M, Capel, P J A and Koene, R A P (1984) Similar activities of IgG1 and IgG2 alloantibodies in enhancement of mouse skin grafts and in opsonization Transplantation 37, 291

Marchesi, V T, Tillack, T W, Jackson, R L, Segrest, J P and Scott, R E (1972) Chemical characterization and surface orientation of the major glycoprotein of the human erythrocyte membrane Proc Natl Acad Sci U S A 69, 1445

Merry A H, Thomson, E E, Rawlinson, V I and Stratton, F (1982) A quantitative antiglobulin test for IgG for use in blood transfusion serology Clin Lab Haematol 4, 393

Ochiai, Y, Furthmayr, H and Marcus, D M (1983) Diverse specificities of five monoclonal antibodies reactive with glycophorin A of human erythrocytes J Immunol 131, 864

Rabellino, E, Colon, S, Grey, H M and Unanue, E R (1971) Immunoglobulins on the surface of lymphocytes I Distribution and quantitation J Exp Med 133, 156

Rearden, A and Masouredis, S P (1980) The antiglobulin reaction with native and enzyme-modified red cells Transfusion 20, 377

Rochna, E and Hughes-Jones, N C (1965) The use of purified ^{125}I-labelled anti-γ-globulin in the determination of the number of D antigen sites on red cells of different phenotypes Vox Sang 10, 675

Shaw, G M, Levy, P C and Lobuglio, A F (1979) Re-examination of the EA rosette assay (Rupley) for Fc receptor leucocytes Clin Exp Immunol 36, 496

Singer, S J (1974) Molecular biology of cellular membranes with applications to immunology Adv Immunol 19, 1

Smith, R S, Longmire, R I, Reid, R T and Farr R S (1970) The measurement of immunoglobulin associated with human peripheral lymphocytes J Immunol 104, 367

Tax, W J M, Willems, H W, Reekers, P P M, Capel, P J A and Koene, R A P (1983) Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells Nature 304, 445

Tax, W J M, Hermes, F F M, Willems, H W, Capel, P J A and Koene, R A P (1984) Fc receptors for mouse IgG1 or human monocytes polymorphism and role in antibody-induced T cell proliferation J Immunol 133, 1185

Van de Winkel, J G J, Tax, W J M, Van Bruggen, M C J, Van Roozendaal, C E P, Willems, H W, Van Duijnhoven J L P, Capel, P J A and Koene, R A P (1987) A new photometric method for the quantitation of Fc receptor for murine IgG1 on human monocytes and cell lines J Immunol Methods 101, 109

CHAPTER 5

SELECTIVE MODULATION OF TWO HUMAN MONOCYTE Fc RECEPTORS FOR IgG BY IMMOBILIZED IMMUNE COMPLEXES

Jan G.J. van de Winkel, Hans L.P. van Duijnhoven, René van Ommen, Peter J.A. Capel, and Wil J.M. Tax

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SELECTIVE MODULATION OF TWO HUMAN MONOCYTE Fc RECEPTORS FOR IgG BY IMMOBILIZED IMMUNE COMPLEXES[1]

JAN G. J. VAN DE WINKEL,[2,3] HANS L. P VAN DUIJNHOVEN, RENÉ VAN OMMEN, PETER J. A. CAPEL,[3] AND WIL J. M. TAX

From the Department of Medicine, Division of Nephrology, University Hospital Nijmegen, Nijmegen, The Netherlands

Two types of IgG FcR, FcRI and FcRII, are constitutively expressed by human monocytes. FcRI (identified by mAb 32.2) binds human (h) IgG, FcRII (identified by mAb IV.3) has a low affinity for hIgG but interacts strongly with murine (m) IgG1. These receptors can be assayed by using indicator E sensitized by hIgG (EA-hIgG) or mIgG1 (EA-mIgG1), respectively. We further characterized these two FcR by modulation studies by using substrate-immobilized immune complexes containing rabbit IgG, goat IgG, or one of the mouse Ig classes or subclasses. After incubating monocytes in microtiter wells containing such immune complexes, binding of the two types of indicator red cells on the apical surface of the monocytes was quantitated using a photometric assay employing the pseudoperoxidase activity of E. No effect on the binding of sensitized E was observed after incubation of monocytes with immune complexes containing mouse IgE, IgA, or IgM, or $F(ab')_2$ fragments of rabbit IgG. High concentrations of immune complexes containing IgG of mouse, rabbit, or goat, however, were able to induce a decrease in binding of both types of sensitized E, suggestive of modulation of both FcRI and FcRII. At lower concentrations of immune complexes, more selective patterns of modulation emerged. Under these conditions, immune complexes containing mIgG1 or mIgG2b, or, surprisingly, goat IgG induced a selective decrease in the binding of EA-mIgG1 (FcRII modulation), while immune complexes containing mIgG2a or rabbit IgG mainly affected the binding of EA-hIgG (FcRI modulation).

By using anti-FcR mAb IV.3, it was confirmed that FcRII was modulated from the apical surface of monocytes after incubation on immune complex coated substrates. Selectivity of FcR-modulation was demonstrated by showing that under these conditions binding of anti-C receptor mAb, and several other anti-monocyte mAb did not decrease.

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[2] Correspondence and reprint requests should be addressed to Jan G J van de Winkel Department of Experimental Immunology State University of Utrecht Catharynesingel 59 3511 GG Utrecht The Netherlands

[3] Present address Department of Experimental Immunology State University of Utrecht Utrecht The Netherlands

Many important biologic effects are mediated by FcR, among which are endocytosis of immune complexes, antibody-dependent cell-mediated cytotoxicity, and the release of biologically active mediators (reviewed by Morgan and Weigle (1)) For a better understanding of these processes, a more detailed knowledge of the structure and function of FcR, as well as their relation with other cell surface receptors is essential

In the last few years knowledge about human monocyte FcR for IgG has progressed rapidly By analyzing the monocyte-dependent induction of human T cell proliferation by murine anti-CD3 antibodies, the presence of two types of FcR was suggested FcRI[4] can interact with mIgG2a and mIgG3 mAb, and proliferation induction is inhibited by hIgG FcRII interacts with mIgG1 mAb and cannot be inhibited by monomeric hIgG (2–4) More information about these FcR has been obtained by the availability of different monoclonal anti-receptor antibodies FcRI has an approximate M_r of 70 kDa and is defined by mAb 32 2, whereas FcRII has an approximate M_r of 40 kDa and is recognized by mAb IV 3 (reviewed by Anderson and Looney (5)) Although immunoprecipitation studies clearly suggest that the two FcR are independent structures, this evidence cannot be regarded as definite since the detergents used to prepare cell lysates might have disturbed an association of these membrane Ag

The relation between different membrane receptors can be studied by using immune complex-coated substrates When monocytes or macrophages are plated on a surface containing immobilized immune complexes, FcR specific for the Fc moiety of the antibody involved move from the apical surfaces of the cells to the area of contact, where they are trapped (6–14) The cells are then no longer capable of FcR-mediated binding (and phagocytosis) at their apical cell surfaces The relation between different cell surface receptors could thus be studied, by using both specific probes for the receptors (e g , sensitized E or anti-receptor antibodies), and different types of ligands CR and FcR have been shown to modulate independently in this type of study both on human monocytes (7, 9, 10) and on murine macrophages (6, 8, 14) It has furthermore been shown that the two types of CR (CR1 and CR3) can be modulated independently on human monocytes (11) For murine macrophages it was

[4] Abbreviations used in this paper FcRI monocyte 72 kDa receptor mediating rosetting with red cells coated by human anti rhesus D IgG FcRII monocyte 40-kDa receptor mediating rosette formation with murine IgG1 sensitized E h human m murine CR C receptor(s) HSA human serum albumin HRBC human E EA antibody sensitized human E

shown that the expression of different types of FcR was regulated selectively after infection of mice with bacillus Calmette Guerin These in vivo observations were further shown to correlate with selective modulation on substrate immobilized immune complexes in vitro (15) For human monocytes similar experiments have not yet been described

In this study we analyzed the specificity of the two types of FcR for IgG on human monocytes by modulation of these receptors by using immobilized immune complexes containing Ig of different species These heterocytophilic interactions with human monocytes are as yet not well defined and are of importance for a better understanding of the in vivo effects of mAb or polyclonal antibodies which are currently used in clinical therapy FcR were detected by using two types of sensitized E EA hIgG and EA mIgG1 (16 17) EA hIgG binding to monocytes was previously shown to be inhibited both by monomeric hIgG and by mIgG2a but not by mIgG2b or mIgG1 EA mIgG1 binding was inhibited by monomeric mIgG1 only and not at all by hIgG mIgG2a or mIgG2b (16) Rosettes were quantitated by using the HRBC associated pseudoperoxidase activity by a sensitive photometric method (18)

Our results show that at high ligand concentrations the two types of FcR for IgG were modulated together whereas at low ligand concentrations selective modulation for the receptors was found

MATERIALS AND METHODS

Monocytes Cytapheresis of healthy volunteers was applied to obtain white blood cell suspensions Monocytes were isolated from these suspensions by Percoll (Pharmacia Uppsala Sweden) centrifugation and counterflow centrifugation as described before (18) Monocyte suspensions consisted for at least 90% of monocytes (evaluated in cytocentrifuge preparations after staining for nonspecific esterase and with May Grunwald Giemsa) and viability was high (>98% assessed by trypan blue exclusion) Purified monocytes were either used immediately or were cryopreserved (19) and their accessory function was studied in mitogenesis assays (3) With respect to mIgG1 and mIgG2b polymorphism of monocyte FcR has been described (3 4 20) Only monocytes which supported the proliferation of human T lymphocytes induced by murine anti CD3 antibodies of the IgG1 and IgG2a subclasses but not of the IgG2b subclass were used for the studies described in this report All experiments presented in this paper have been performed by using cryopreserved monocytes but were repeated at least once using freshly isolated monocytes which yielded similar results

Antibodies Anti DNP hybridoma IGEL b4 (mIgE) (21) was obtained from American Type Culture Collection (Rockville MD) An $(NH_4)_2SO_4$ precipitate of culture supernatant was used in the experiments Purified myeloma protein MOPC 315 (mIgA) reactive with DNP (22) was obtained from Litton Bionetics (Kensington MD) Anti DNP mAb MAS 057 (mIgM) (23) was obtained as ascites from Sera lab (Sussex UK) Anti DNP mAb D1G10 (mIgG1) and D1B12 (mIgG2b) were produced in our own laboratory after immunization of BALB/c mice with DNP keyhole limpet hemocyanin and by fusion with Sp2/0 cells Culture supernatants of these mAb were used for the construction of surface immobilized immune complexes A rabbit hyperimmune anti DNP serum was provided by Dr Lems (Department of Medicine Division of Nephrology Nijmegen The Netherlands) and the IgG fraction of goat anti DNP hyperimmune serum by Dr de Vries (Department of Medicine Division Rheumatology Nijmegen) Ascites of myeloma protein 5T2 (mIgG2a) (24) reactive with DNP was provided by Dr Radl (TNO Rijswijk The Netherlands) and was purified by $(NH_4)_2SO_4$ precipitation followed by different chromatographic procedures (25) Anti BSA mAb 6007 11 (mIgG1) was produced by Dr Van de Broek (Department of Medicine Division of Rheumatology Nijmegen) and was used as ascites The IgG fraction of hyperimmune rabbit anti BSA serum was obtained from DAKO (Copenhagen Denmark) and the IgG fraction of hyperimmune goat anti BSA antiserum from Sigma Chemical Co (St Louis MO) Anti HSA antibodies produced in rabbits were obtained as purified whole IgG and as $F(ab')_2$ fragments from Cappel (Malvern PA) Purified antibodies (and antibody fragments) were analyzed by immunoelectrophoresis and double diffusion in agarose No residual antibodies of a contaminating (sub)class could be detected (i e more than 99 9% pure) The antibody classes and subclasses were further confirmed by an FIISA (26) by using (sub)class specific antibodies from both Nordic (Tilburg The Netherlands) and Meloy (Springfield VA)

A mIgG1 anti human glycophorin A mAb was used to sensitize HRBC (4) The hybridoma producing this antibody was provided by M Bos (CLB Amsterdam The Netherlands) To sensitize the HRBC with human IgG we used a human alloantiserum against rhesus D obtained from Merz & Dade (Dudingen Switzerland)

mAb OKM1 directed against CR3 (27) and OKM5 against membrane Ag p88 (28) were obtained from Ortho Diagnostics (Raritan NJ) mAb anti Leu M3 against a monocyte/macrophage specific Ag p55 (Clone MφP 9) (29) anti Leu M5 reacting with p150 95 (30) and anti CR1 (Clone 44D) (31) were obtained from Becton Dickinson (Mountain View CA) Culture supernatant of mAb IV 3 (anti 40 kDa FcR) (17) was a generous gift of Dr Clark Anderson (Ohio State University Columbus OH)

Immobilization of immune complexes Flat bottomed microtiter plates (Costar Cambridge MA) were supplemented with 100 μl of either BSA (Cohn fraction V Sigma) BSA DNP (Calbiochem Behring La Jolla CA) or HSA (Merieux Lyon France) diluted in PBS to concentrations varying from 0 1 μg/ml to 1 mg/ml and incubated overnight at 4°C After three washes either 100 μl PBS (control) or 100 μl of a saturating concentration of antibody were added to each well for 1 h at room temperature After incubation the plates were washed thrice and were used immediately

Saturating concentrations of antibodies were determined in a sensitive ELISA (26) by using high concentrations of coated Ag (0 5 to 1 0 mg/ml) The saturating concentrations were routinely used except for some experiments in which the degree of substrate sensitization was varied (see *Results*) Furthermore at these concentrations no contaminating Ig of another (sub)class could be detected by FLISA by using (sub)class specific antibodies (26) in any of the antibody preparations Culture supernatants of hybridomas were used when available Otherwise we used either ascites or purified Ig which yielded similar results

Sensitization of E Sensitization of HRBC was performed as we described before (18) Briefly 0 5% suspensions of HRBC were incubated with serial dilutions of anti HRBC antibodies (or as a control for nonspecific binding with PBS alone) for 30 min at 37°C After incubation sensitized (and as a control unsensitized) HRBC were washed resuspended at 0 5% in RPMI 1640 containing 5% FCS and used within 30 min

Quantitative EA rosette assay Control or immune complex coated microtiter wells were supplemented with 100 μl of monocyte suspensions (4×10^6 cells/ml in RPMI 1640 with 10% FCS) and incubated for 90 min at 37°C After incubation nonadherent cells were washed away and FcR expression was determined on monocyte monolayers as we described before (18) Briefly sensitized HRBC were dispensed into the microtiter plates and incubated for 1 h at 4°C The extent of EA rosetting was quantitated by using the E associated pseudoperoxidase activity measured photometrically at 450 nm A binding index was calculated for each experiment (to correct for variation in background absorbance values) as follows

$$[1 - (E_{450}/EA_{450})] \times 100 = \text{binding index}$$

E_{450} Extinction at 450 nm in wells to which HRBC *without* sensitizing antibody were added

EA_{450} Extinction at 450 nm in wells to which *sensitized* HRBC were added

Previously we demonstrated the Fc dependency of EA rosetting by showing that HRBC sensitized with (high concentrations) of $F(ab')_2$ fragments of anti glycophorin A antibody did not cause a positive signal in the photometric assay (18) The HRBC suspensions sensitized with different dilutions of antisera were always tested in duplicate duplicate values deviated less than 10%

ELISA for monocyte membrane Ag Monocytes (4×10^5 cells/well) were incubated for 30 min at 37°C in microtiter plates which had been previously coated first with BSA (10 μg/ml) then with poly L lysine (Sigma 100 μg/ml) and finally with either rabbit anti BSA IgG or PBS (as a control) After centrifugation (5 min at 200 × *g*) the cells were fixed in 0 025% glutaraldehyde (20 min at room temperature) washed by vertical immersion in warm PBS (37°C) and to avoid nonspecific binding the plates were coated with a 1% solution of gelatin (Difco Laboratories Detroit MI) in PBS (1 h at 37°C) After washing saturating amounts of mAb were added to duplicate wells and incubated (45 min at 37°C) After incubation and washing peroxidase conjugated goat anti mouse L and H chain antibodies

(Nordic Tilburg the Netherlands) were added and incubated for 45 min at 37°C The plates were washed and the amount of bound antibodies was quantitated by adding a substrate solution containing 5'-aminosalicylic acid (26) and after 30 min the extinction was determined at 450 nm in a Titertek Multiscan

RESULTS

Incubation of monocytes with immobilized immune complexes can affect the binding of sensitized HRBC FcRI and FcRII can be independently detected by EA-rosetting by using EA-hIgG or EA-mIgG1, respectively (16). When monocytes were incubated in microtiter wells containing a high concentration of immune complexes of HSA and rabbit IgG anti-HSA, the binding of both EA-hIgG and EA-mIgG1 indicator cells (sensitized with different amounts of antibodies) was strongly decreased in comparison to monocytes incubated in wells containing only HSA No such decrease in EA-rosetting was observed when $F(ab')_2$ fragments of rabbit anti-HSA IgG were used (Fig 1) These findings suggest that the diminution in binding of sensitized HRBC is dependent on interaction of monocytes with the Fc-moiety of IgG in immune complexes. This conclusion was supported by the experiments summarized in Table I The extent of indicator HRBC binding is presented here only at one (maximal) level of HRBC sensitization (i e., at the edge of binding-plateau) and Ag were coated by using a concentration of 100 μg/ml The table shows that no decrease in binding of sensitized HRBC was observed when monocytes were plated in immune complex-coated wells containing either mIgE, mIgA, or mIgM Similar results were obtained with higher concentrations (>100 μg/ml) of coated Ag (results not shown) Monocyte monolayers which had been incubated on immune complex-coated substrates containing any of the murine IgG subclasses, displayed a decreased binding of both types of indicator cells A similar decreased binding was observed for immune complexes containing different rabbit or goat IgG antibodies These data indicate that, when immobilized immune complexes were present in high concentrations, both types of FcR apparently had a similar pattern of reactivity

Incubation of monocytes with immobilized immune complexes selectively affects FcR expression We next studied whether the incubation of monocytes with immobilized immune complexes indeed affects the expression of FcR, and furthermore, whether this effect is specific or involves nonspecific co-modulation of monocyte membrane Ag For this purpose, we used a panel of mAb, including mAb IV 3, which is directed against FcRII on human monocytes (17), and which we previously found to inhibit EA-rosetting between these cells and EA-mIgG1 for over 80%, whereas EA-rosetting with EA-hIgG remained unaffected (16) Table II shows that by incubating human monocytes on rabbit anti-BSA IgG containing immune complex-coated substrates, binding of mAb IV 3 decreased by about 50% In parallel experiments we observed (under identical conditions) a decrease of approximately 70% in binding of both types of indicator cells to the monocytes This reduction is presumably due to the modulation of FcR from the apical surface of monocytes to the substrate-adherent part of their membrane, as was also shown in the murine system (12 14) Another mAb, 32.2 (32) directed against FcRI on human monocytes (generously provided by Dr Clark Anderson, Ohio State University), was also tested but yielded too low a signal in our assay system and could not be used for these studies The results in Table II show that interaction of monocytes with immune complex-coated surfaces does not induce redistribution of unrelated monocyte membrane proteins CR1 and CR3 on human monocytes did not co-modulate with FcRII, nor did the monocyte membrane Ag detected by anti-Leu-M5 (p 150,95), anti-Leu-M3 (p 55), and OKM5 (p 88)

Effect of ligand concentration on modulation of FcR. Both for human (9, 10) and murine (6, 12) monocytes/macrophages the extent of FcR modulation is clearly dependent on the amount of substrate-immobilized immune complexes At high concentrations of immune complexes (Ag coating at 100 μg/ml or more), no selective effect on the modulation of the two FcR was observed When the concentration of Ag (and therefore the amount of immune complexes) was lowered, however, selective patterns of modulation emerged, as is illustrated in Figure 2 for the mIgG2a mAb 5T2 The amount of DNP-BSA used for coating was varied (100 μg, 5 μg or 0 5 μg/ml) and subsequently a saturating amount of the anti-DNP antibody was added At lower immune complex concentrations, FcRI was gradually modulated more strongly than FcRII When Ag was coated at a fixed concentration of 10 μg/ml and the amount of complexing anti-DNP mAb was varied, similar results were obtained (data not shown).

When mAb of three different murine IgG isotypes were tested in modulation studies at varying Ag concentrations, two different patterns were observed as is shown in Figure 3 The extent of indicator HRBC binding is presented only at a maximal level of HRBC-sensitization,

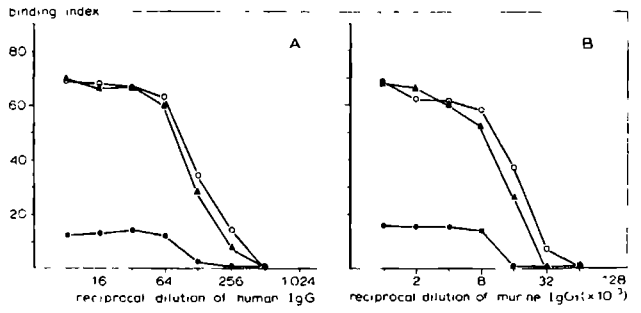

Figure 1 Modulation of two types of FcR on human monocytes by surface-bound immune complexes Monocytes were cultured in microtiter wells coated with HSA only (100 μg/ml ○) or in wells coated with HSA and subsequently incubated with a saturating amount of either $F(ab')_2$ fragments (▲) or whole IgG (●) of rabbit anti-HSA antibody After incubation the expression of FcR was assayed photometrically by using EA-hIgG (*A*) or EA mIgG1 (*B*) sensitized at different dilutions of antibodies Results are representative for three experiments

TABLE I

Binding of sensitized E to monocytes incubated on different immune complex coated substrates[a]

Substrate Coated with		Binding of Sensitized HRBC (% of Control)	
Immune complex	Ig class/subclass	EA higG	EA migG1
BSA DNP (IGFL b4)[b]	migE	102 ± 2[c]	91 ± 9
BSA DNP (MOPC 315)	migA	96 ± 13	103 ± 2
BSA DNP (MAS 057)	migM	95 ± 11	102 ± 2
BSA DNP (5T2)	migG2a	5 ± 3	8 ± 11
BSA DNP (D1B12)	migG2b	22 ± 11	4 ± 5
BSA DNP (D1G10)	migG1	28 ± 11	20 ± 3
BSA (6007 11)	migG1	17 ± 11	17 ± 5
BSA (rabbit anti BSA)	rigG[d]	3 ± 5	11 ± 8
BSA DNP (rabbit anti DNP)	rigG	13 ± 12	20 ± 5
BSA (goat anti BSA)	gigG[e]	15 ± 11	4 ± 8
BSA DNP (goat anti DNP)	gigG	11 ± 8	6 ± 7

[a] Monocytes were incubated in microtiter wells coated with BSA or BSA DNP (100 μg/ml) only (control wells) or in wells which had been coated with BSA/BSA DNP and subsequently incubated with an optimal concentration of the indicated antibodies (immune complex wells). After incubation the binding of different indicator HRBC was quantitated by using their pseudoperoxidase activity as described in *Materials and Methods*. The results are expressed as percent of control binding calculated by the following formula

$$\left[\frac{EA_{450}\ (\text{immune complex}) - E_{450}\ (\text{immune complex})}{EA_{450}\ (\text{control}) - E_{450}\ (\text{control})}\right] \times 100$$

[b] Substrate coating with the antibody name in parentheses.

[c] Results represent the mean ± SD of three or more individual experiments.

[d] r = rabbit.

[e] g = goat.

TABLE II

Immobilized immune complexes decrease the expression of monocyte FcR but not of other membrane proteins[a]

mAb	Ag	Relative Ag Expression
IV 3	FcRII	0 54 ± 0 09 (6)[b]
Anti CR1	CR1	1 22 ± 0 35 (6)
OKM1	CR3	1 09 ± 0 11 (6)
Anti Leu M5	p 150 95	1 09 ± 0 17 (6)
Anti Leu M3	p 55	1 03 ± 0 08 (3)
OKM5	p 88	1 02 ± 0 18 (6)

[a] Microtiter plates were coated with 10 μg/ml BSA followed by PBS (control) or rabbit anti BSA IgG (immobilized immune complex). Monocytes were incubated and binding of the indicated mAb was assayed by FLISA as described in *Materials and Methods*.

[b] Results represent the ratio of the FLISA signal obtained with monocytes that had been incubated with immobilized immune complexes relative to the signal from monocytes incubated in the control wells. Experiments were performed in duplicate wells and the mean ± SD of three to six experiments (as indicated in parentheses) is given.

as in Table I. An essential difference between Table I and Figure 3 is the concentration of immune complexes at which the experiments were performed: a high Ag concentration (100 μg/ml) for Table I, and low concentrations (0.5 to 1 μg/ml) for Figure 3. As was also shown in Figure 2, at low Ag concentrations migG2a mAb 5T2 reproducibly modulated FcRI stronger than FcRII. A different pattern was found for migG1 mAb D1G10, which shows greater selectivity for the FcR assayed by EA-migG1 (FcRII). Comparable results were obtained with a migG1 mAb with reactivity for BSA. This mAb (6007-11) modulated FcRII by 51% (mean of three experiments), whereas FcRI was not modulated at low Ag concentrations. By using mAb D1B12 (migG2b), FcRI was modulated by approximately 40%, whereas under the same conditions FcRII disappeared almost completely (for about 97%, Fig. 3), indicating that migG2b antibodies interact preferentially with FcRII (as was also found for migG1).

Two modulation patterns were observed when the effects of rabbit and goat IgG in immune complex form were evaluated (Fig. 4). Rabbit IgG anti-BSA selectively modulated FcRI. Similar results were obtained by using a rabbit anti-BSA-DNP antibody which modulated FcRI by 48% (mean of four experiments), whereas FcRII remained unaffected. By contrast, goat IgG anti-BSA, reproducibly affected the binding of EA-migG1 (FcRII) more strongly than the binding of EA higG (FcRI). This effect was also observed when a goat anti-BSA DNP antibody was tested (63% modulation of EA-migG1 binding, and no modulation of FcRI, mean of three experiments).

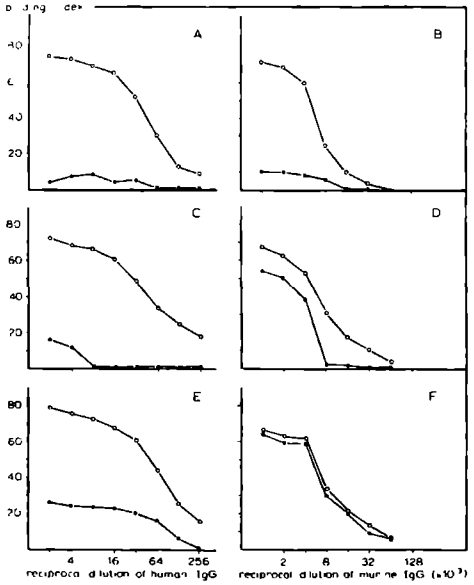

Figure 2. Effect of substrate sensitization on modulation of FcR on human monocytes. Monocytes were incubated in microtiter wells coated with DNP BSA only (○) in concentrations of 100 μg/ml (*A, B*), 5 μg/ml (*C, D*) or 0.5 μg/ml (*E, F*) or in wells coated with BSA DNP and subsequently incubated with migG2a anti DNP mAb (5T2 ●). After incubation FcR expression was determined by using EA higG (*A, C, E*) or EA migG1 (*B, D, F*) as indicator cells in the photometric assay. Results are representative for four experiments.

Our results illustrate that not only the degree of FcR-modulation was strictly dependent on the amount of substrate-immobilized immune complexes, but that this parameter had also an important effect on the apparent selectivity of modulation of FcR on human monocytes.

DISCUSSION

Human monocytes constitutively possess two types of FcR for IgG, FcRI and FcRII (reviewed by Anderson and Looney (5)). These receptors can be studied in rosette assays by using EA-higG and EA migG1, respectively, as indicator cells (16, 17). We have previously shown that at least 30 times more migG1 molecules than higG molecules have to be present per erythrocyte for half-maximal rosetting to occur with monocytes which suggest that there is a large difference in affinity between the two types of FcR (33). Previous studies on the inhibition

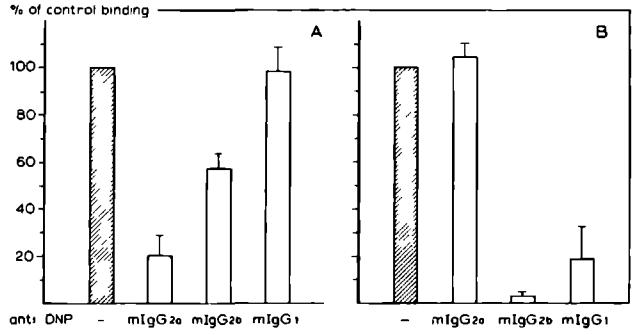

Figure 3 Effect of substrate immobilized immune complexes containing different murine IgG subclasses on the modulation of FcR on human monocytes Monocytes were incubated in wells coated with DNP BSA only (control) or in wells which had been coated with DNP BSA and subsequently with either mIgG2a mAb (5T2 DNP BSA 0 5 μg/ml) mIgG2b mAb (D1B12 DNP BSA 0 5 μg/ml) or mIgG1 mAb (D1G10 DNP BSA 1 μg/ml) Control wells had been coated with DNP BSA at 0 5 μg/ml 0 5 μg/ml and 1 μg/ml respectively Binding of HRBC sensitized with either hIgG (*A*) or mIgG1 (*B*) was quantitated using the photometric assay Results are expressed as per cent of control binding as described in Table I The means ± SD of three individual experiments are presented

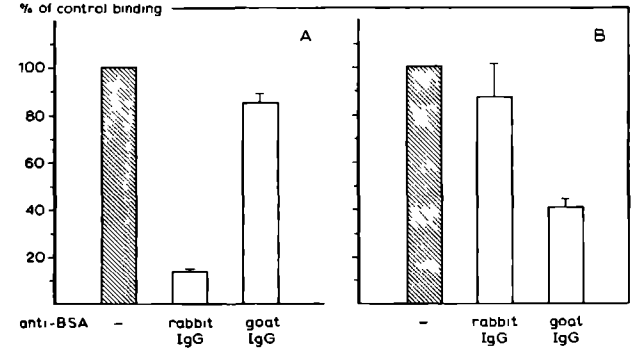

Figure 4 Effect of rabbit and goat IgG in surface bound immune complexes on the modulation of FcR on human monocytes Monocytes were incubated in wells coated with BSA only (control) or in wells which had been coated with BSA and subsequently with either rabbit IgG anti BSA (BSA 0 1 μg/ml) or goat IgG anti BSA (BSA 10 μg/ml) Control wells had been coated with BSA at 0 1 μg/ml and 10 μg/ml respectively Binding of EA hIgG (*A*) and EA mIgG1 (*B*) was quantitated photometrically Results are expressed as percent of control binding as described in Table I Results show the mean ± SD of three individual experiments

of rosetting by monomeric Ig and anti-FcR mAb (16, 17) have suggested that the two FcR are independent In the present study we investigated the specificity of the different FcR for Ig of different species by using modulation of FcR by immobilized immune complexes containing a defined (sub)class of Ig

When we plated human monocytes in immune complex-coated microtiter plates containing relatively high concentrations of immune complexes, binding of both EA-hIgG and EA-mIgG1 was virtually abolished This effect was only observed in plates coated with IgG-containing immune complexes, and was dependent on the presence of the Fc-moiety since immune complexes containing $F(ab')_2$ fragments were ineffective Furthermore, when monocytes were incubated with immune complexes containing either mIgA, mIgE, or mIgM antibodies, rosette formation was not affected Since receptors for IgE (34), IgA (35), and IgM (36) have previously been described on human monocytes, these results indicate that the binding sites of these Ig are located on structures different from those for IgG The existence of different FcR for the various classes of Ig as independent structures was previously also suggested by inhibition studies performed with both murine macrophages (37) and human monocytes (34–36)

The incubation of monocytes with immobilized immune complexes induced a decrease in binding of anti-FcRII mAb IV 3 as shown in Table II, although the decrease in binding of this mAb was less as compared with the decrease in binding of EA-mIgG1 in parallel experiments Similar results have been described in the mouse macrophage model by using mAb 2 4G2 (12–14) As discussed by Michl et al (13) anti-FcR antibodies may have access to FcR at sites that are no longer accessible to sensitized erythrocytes The decreased binding of mAb IV 3 in our model supports the concept that modulation of FcR is indeed responsible for the observed decrease in binding of sensitized E A shift of FcR from the apical site to the substrate-adherent part of the cells during incubation on immobilized immune complexes is the most likely explanation for this type of modulation (8, 12–14) Although anti-FcR mAb have been frequently used for modulation studies, Michl et al (13) showed that several processes may contribute to decreased binding of anti-FcR mAb and concluded that "only E(IgG) binding provides an accurate assessment of the rate of FcR removal from macrophages' upper surface " Our experiments also indicate that incubation of monocytes on immobilized immune complexes did not cause a general redistribution of membrane antigens FcRII was modulated independently of both types of CR on human monocytes, as has been described previously also for FcRI (7, 9, 10) The experimental findings are most likely explained by a random movement of FcR within the membrane of monocytes, and the effect of substrate-adherent immune complexes is the trapping of FcR diffusing into

the segment of the plasma membrane, which is in contact with the substrate

Because a stoichiometric relationship between the number of IgG molecules bound to immobilized Ag and the extent of FcR-modulation was observed for murine macrophages (12), we studied the effect of this parameter in our system By varying the amount of coated Ag, the concentration of immobilized immune complexes could be manipulated Different patterns of modulation were revealed for the various types of Ig, when modulation was studied on immune complex-coated substrates containing different amounts of ligands At low concentrations of immune complexes, antibodies of the mIgG2a isotype selectively modulated FcRI, and immune complexes containing mIgG1 or mIgG2b showed selectivity for FcRII Immune complexes containing mIgG2b, however, also modulated FcRI to a significant extent (at low Ag concentrations) Comparable results have been obtained by Ezekowitz et al (15) studying mouse macrophages, who reported that incubation of macrophages with substrate-immobilized immune complexes containing mIgG2b strongly inhibited the binding of mIgG2b-sensitized E but also had a significant inhibitory effect on the binding of mIgG2a-sensitized E Rabbit IgG exhibited specificity for FcRI in the modulation experiments, whereas goat IgG was surprisingly found to modulate FcRII to a greater extent than FcRI.

All the data in this work can be explained by assuming that both types of FcR have affinity for all of the IgG ligands we tested Another assumption which also has to be made is that differences in affinity exist between the two FcR Each FcR will display the highest affinity for the homologous ligand, and a much lower affinity for heterologous ligands Experimental evidence to support this hypothesis has been described for both murine macrophages (38, 39) and human cells of monocyte/macrophage origin (40) With the human monocytic cell line U937, binding of two types of ligands (mIgG2a and aggregated mIgG2b) was inhibited 30 to 135 times more efficiently by the homologous ligand than by the heterologous one (40) On the basis of these results, of studies with anti-FcRII mAb IV.3, and of experiments studying trypsin sensitivity of binding, Jones et al (40) proposed two different FcR for IgG on human monocytic cells In our opinion, the observed cross-reactivities can only be explained by extensive homologies between the different types of ligands Strong homologies have indeed been described for the different murine IgG subclasses (41, 42) Extensive homology was also found between rabbit and mouse $CH\gamma$ fragments (14)

As a consequence of the high affinity of FcR for immune complex-coated substrates these cross-reactivities are amplified and cause the observed co-modulation of the two types of FcR at relatively high concentrations of immobilized immune complexes. Differences in the concentration of immobilized immune complexes may also explain the conflicting results which have been reported for FcR-modulation on murine macrophages Sung (14) reported receptor co-modulation on macrophages plated on different Ag-antibody complexes, whereas Ezekowitz et al (15) clearly demonstrated selective modulation of FcR on murine macrophages

An efficient interaction of murine IgG2b antibodies with human monocyte FcR has previously been reported to be absent both when binding of monomeric and polymeric forms were tested (16, 40, 43). Only under conditions of low ionic strength a significant mIgG2b aggregate binding to FcRII on human cell lines was observed (40) Our results support those of Jones et al. (40), and furthermore accentuate the high affinity of FcR for Ag-bound IgG Whether the interaction observed between FcRII and mIgG2b-containing immune complexes is amplified by changes in ionic strength of the medium under the conditions employed is not known However, since FcR have previously been shown to mediate ion channel activity (44), the concentration of FcR at the substrate-adherent part of the monocytes is probably high and the volume of the membrane-substrate interface very small, this possibility may not be too farfetched.

An efficient interaction between Fc and FcR under these conditions was also suggested by the observed decrease in binding of the two types of indicator HRBC by monocytes incubated on immune complexes containing goat IgG, since goat IgG in monomeric form interacts very poorly with human monocyte FcR (45) Furthermore, when modulation of FcR was studied on substrates, coated with goat IgG in a non-Ag specific way, no effects were found (46) In both these studies only FcRI was assayed The observed preference of goat IgG for FcRII was also found for immune complexes containing polyclonal rat IgG (results not shown). This indicates that FcRII does not interact exclusively with mIgG1 antibodies. The isotype(s) of rat IgG mediating the interaction with FcRII are currently studied in our laboratory by using different rat mAb

In conclusion, we demonstrate here that the two types of FcR for IgG on human monocytes can be modulated selectively on substrate-immobilized immune complexes. The pattern of modulation was found to be critically dependent, both on the type of immobilized ligand, and on the amount of immobilized immune complexes We furthermore conclude that the two FcR are independent from each other (and from CR). FcRI interacts primarily with hIgG and cross-reacts with mIgG2a, FcRII has a low affinity for monomeric hIgG but interacts strongly with mIgG1. Both types of receptors also appear to have affinity, although much lower, for other (sub)classes of IgG.

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REFERENCES

1 **Morgan, E. L., and W. O. Weigle** 1987 Biological activities residing in the Fc region of immunoglobulin *Adv. Immunol. 40 61*
2 **Looney, R. J., and G. N. Abraham** 1984 The Fc portion of intact IgG blocks stimulation of human PBMC by anti-T3 *J. Immunol. 133 154*
3 **Tax, W. J. M., H. W. Willems, P. P. M. Reekers, P. J. A. Capel, and R. A. P. Koene** 1983 Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells *Nature 304 445*
4 **Tax, W. J. M., F. P. M. Hermes, H. W. Willems, P. J. A. Capel, and R. A. P. Koene** 1984 Fc receptors for mouse IgG1 on human monocytes: polymorphism and role in antibody induced T cell proliferation *J. Immunol. 133 1185*
5 **Anderson, C. L., and R. J. Looney** 1986 Human leukocyte IgG Fc

receptors *Immunol Today 7 264*

6 **Rabinovitch M R E Manejias and V Nussenzweig** 1975 Selective phagocytic paralysis induced by immobilized immune complexes *J Exp Med 142 827*

7 **Douglas S D** 1976 Human monocyte spreading in vitro Inducers and effects on Fc and C3 receptors *Cell Immunol 21 344*

8 **Michl J M M Pieczonka J C Unkeless and S C Silverstein** 1979 Effects of immobilized immune complexes on Fc and Complement receptor function in resident and thioglycollate elicited mouse peritoneal macrophages *J Exp Med 150 607*

9 **Ragsdale C G and W P Arend** 1980 Loss of Fc receptor activity after culture of human monocytes on surface bound immune complexes *J Exp Med 151 32*

10 **Arend W P and R J Massoni** 1981 The effect of complement in adherent immune complexes on Fc and C3 receptor expression in human monocytes *Immunology 44 717*

11 **Wright S D and S C Silverstein** 1982 Tumour promoting phorbol esters stimulate C3b and C3b receptor mediated phagocytosis in cultured human monocytes *J Exp Med 156 1149*

12 **Michl J J C Unkeless M M Pieczonka and S C Silverstein** 1983 Modulation of Fc receptors of mononuclear phagocytes by immobilized antigen antibody complexes *J Exp Med 157 1746*

13 **Michl J M M Pieczonka J C Unkeless G I Bell and S C Silverstein** 1983 Fc receptor modulation in mononuclear phagocytes maintained on immobilized immune complexes occurs by diffusion of the receptor molecule *J Exp Med 157 2121*

14 **Sung S-S J** 1985 Phagocytosis by mouse peritoneal macrophages plated on monoclonal antibody coated immune complex substrates effects of complexes of different IgG subclasses on Fc receptor functions *J Immunol 135 1981*

15 **Ezekowitz R A B M Bampton and S Gordon** 1983 Macrophage activation selectivity enhances expression of Fc receptors for IgG2a *J Exp Med 157 807*

16 **Van de Winkel J G J W J M Tax M C J van Bruggen C E P van Roozendaal H W Willems A Vlug P J A Capel and R A P Koene** 1987 Characterization of two Fc receptors for mouse immunoglobulins on human monocytes and cell lines *Scand J Immunol 26 663*

17 **Looney R J G N Abraham and C L Anderson** 1986 Human monocytes and U937 cells bear two distinct Fc receptors for IgG *J Immunol 137 1641*

18 **Van de Winkel J G J W J M Tax M C J van Bruggen C E P van Roozendaal H W Willems J L P van Duijnhoven P J A Capel and R A P Koene** 1987 A new photometric method for the quantitation of Fc receptors for murine IgG1 on human monocytes and cell lines *J Immunol Methods 101 109*

19 **Shah V O D L McCarley and R S Weiner** 1984 Antibody dependent cell mediated cytotoxicity of cryopreserved human monocytes *Cryobiology 21 475*

20 **Tax W J M H Spits H F M Hermes H W Willems P J A Capel and R A P Koene** 1985 Polymorphism of human Fc receptors for mouse IgG1 and IgG2b *Transplant Proc 17 794*

21 **Rudolph A K P D Burrows and M R Wabl** 1981 Thirteen hybridomas secreting hapten specific immunoglobulin E from mice with Ig^a or Ig^b heavy chain haplotype *Eur J Immunol 11 527*

22 **Eisen H N E S Simms and M Potter** 1968 Mouse myeloma proteins with antihapten antibody activity The protein produced by plasma cell tumour MOPC 315 *Biochemistry 7 4126*

23 **Jasani B D W Thomas and E Williams** 1981 Use of monoclonal antihapten antibodies for immunolocalization of tissue antigens *J Clin Pathol 34 1000*

24 **Radl J J W Croese C Zurcher M H M Van den Enden Vieveen R J Brandijk M Kazil J J Haayman P H Reitsma and O L M Bijvoet** 1985 Influence of treatment with APD Biphosphonate on the bone lesions in the mouse 5T2 multiple myeloma *Cancer 55 1030*

25 **Lems S P M T P M Rijke Schilder P J A Capel and R A P Koene** 1984 Similar activities of IgG1 and IgG2 alloantibodies in enhancement of mouse skin grafts and in opsonization *Transplantation 37 291*

26 **Faaber P T P M Rijke L B A van de Putte P J A Capel and J H M Berden** 1986 Cross reactivity of human and murine anti DNA antibodies with heparan sulphate *J Clin Invest 77 1824*

27 **Breard J E L Reinherz P C Kung G Goldstein and S F Schlossman** 1980 A monoclonal antibody reactive with human peripheral blood monocytes *J Immunol 124 1943*

28 **Talle M A P E Rao E Westberg N Allegar M Makowski R S Mittler and G Goldstein** 1983 Patterns of antigenic expression on human monocytes as defined by monoclonal antibodies *Cell Immunol 78 83*

29 **Dimitriu Bona A G R Burmester S J Waters and R J Winchester** 1983 Human mononuclear phagocyte differentiation antigens *J Immunol 130 145*

30 **Lanier L L M A Arnaout R Schwarting N L Warner and G D Ross** 1985 P150/95 third member of the LFA 1/CR3 polypeptide family identified by anti Leu M5 monoclonal antibody *Eur J Immunol 15 713*

31 **Iida K R Mornaghi and V Nussenzweig** 1982 Complement receptor (CR1) deficiency in erythrocytes from patients with systemic lupus erythematosus *J Exp Med 155 1427*

32 **Anderson C L P M Guyre J C Whitin D H Ryan R J Looney and M W Fanger** 1986 Monoclonal antibodies to Fc receptors for IgG on human mononuclear phagocytes antibody characterization and induction of superoxide production in a monocyte cell line *J Biol Chem 162 12856*

33 **Van de Winkel J G J W J M Tax A Groeneveld W P M Tamboer P H M de Mulder and P J A Capel** 1988 A new radiometric assay for the quantitation of surface bound IgG on sensitized erythrocytes *J Immunol Methods 108 95*

34 **Melewicz F M and H L Spiegelberg** 1980 Fc receptors for IgE on a subpopulation of human peripheral blood monocytes *J Immunol 125 1026*

35 **Fanger M W L Chen J Pugh and G M Bernier** 1980 Subpopulations of human peripheral granulocytes and monocytes express receptors for IgA *Proc Nat Acad Sci USA 77 3640*

36 **Haegert D G** 1979 Phagocytic peripheral blood monocytes from rabbits and humans express membrane receptors specific for IgM molecules evidence that incubation with neuraminidase exposes cryptic IgM (Fc) receptors *Clin Exp Immunol 35 484*

37 **Vetvicka V L Fornusek P W Kincade and J Kopecek** 1986 Co-expression of different types of Fc receptors on murine peritoneal macrophages *Eur J Immunol 16 901*

38 **Heusser C H C L Anderson and H M Grey** 1977 Receptors for IgG subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line *J Exp Med 145 1316*

39 **Haeffner-Cavaillon N M Klein and K J Dorrington** 1979 Studies on the Fc γ receptor of the murine macrophage like cell line P388D1 *J Immunol 123 1905*

40 **Jones D H R J Looney and C L Anderson** 1985 Two distinct classes of IgG Fc receptors on a human monocyte line (U937) defined by differences in binding of murine IgG subclasses at low ionic strength *J Immunol 135 3348*

41 **Francus T and B K Birshtein** 1978 An IgG2a producing variant of an IgG2b producing mouse myeloma cell line Structural studies on the Fc region of parent and variant heavy chains *Biochemistry 17 4324*

42 **Diamond B L Boccumini and B K Birshtein** 1985 Site of binding of IgG2b and IgG2a by mouse macrophage Fc receptors by using cyanogen bromide fragments *J Immunol 134 1080*

43 **Lubeck M D Z Steplewski F Baglia M H Klein K J Dorrington and H Koprowski** 1985 The interaction of murine IgG subclass proteins with human monocyte Fc receptors *J Immunol 135 1299*

44 **Young J D-E J C Unkeless T M Young A Mauro and Z A Cohn** 1983 Role for mouse macrophage IgG Fc receptor as ligand dependent ion channel *Nature 306 186*

45 **Woof J M L J Partridge R Jefferis, and D R Burton** 1986 Localisation of the monocyte binding region on human immunoglobulin G *Mol Immunol 23 319*

46 **Jungi T W G van Below P G Lerch and P J Spaeth** 1987 Modulation of human monocyte Fc receptor function by surface absorbed IgG *Immunology 60 261*

CHAPTER 6

ACTIVITY OF TWO TYPES OF Fc RECEPTORS, FcγRI AND FcγRII, IN HUMAN MONOCYTE CYTOTOXICITY TO SENSITIZED ERYTHROCYTES

Jan G.J. van de Winkel, Ger J.J.C. Boonen, Peter L.W. Janssen, Arjen Vlug, Nancy Hogg, and Wil J.M. Tax

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SUMMARY

Human monocytes constitutively express two types of Fc receptors for IgG, FcγRI and FcγRII. FcγRI has a high affinity for human IgG, whereas FcγRII interacts poorly with human IgG, but cross-reacts with mouse IgG1. Previous studies showed furthermore that FcγRII is polymorphic. Monocytes from different individuals can either have a high, or weak interaction with mouse IgG1 (high-, respectively low-responder individuals; HR or LR).

We have investigated the cytotoxicity of human monocytes to two types of sensitized erythrocytes. Erythrocytes sensitized with human IgG (EA-human IgG) were used to assay FcγRI function, and erythrocytes sensitized with mouse IgG1 (EA-mouse IgG1) were used to assay FcγRII. Both types of FcγR were observed to mediate ADCC, which was further characterized by using different monoclonal anti-FcγR antibodies and monomeric IgG's. Lysis of EA-human IgG was inhibited by both monomeric human IgG and mouse IgG2a in a dose-dependent way and also by anti-FcγRI mAb 10.1. Cytolysis of EA-mouse IgG1 was inhibited by monomeric mouse IgG1 and by two anti-FcγRII mAb, IV.3 and CIKM5. Antibodies of the mouse IgG2b isotype affected neither type of ADCC. The effectivity of cytotoxicity mediated by either of the FcγR was studied using targets sensitized with a calibrated number of IgG molecules, and was found to differ dramatically. At least 20 times more IgG molecules per target cell were necessary to obtain half-maximal cytotoxicity mediated by FcγRII than for FcγRI-mediated cytolysis. Furthermore, HR monocytes were more effective than LR monocytes in mediating FcγRII-dependent cytotoxicity. These studies demonstrate that FcγRII can mediate ADCC, although a higher degree of target cell sensitization is required than for FcγRI-mediated ADCC.

1. INTRODUCTION

Unstimulated human monocytes express two types of receptors for the Fc moiety of IgG (FcγR). The first type, FcγRI, has an approximate molecular weight of 70 kDa and binds human IgG as well as mouse IgG2a and rabbit IgG with high affinity. FcγRII has an approximate molecular weight of 40 kDa, interacts poorly with aggregated human IgG, has no detectable affinity for monomeric human IgG, and cross-reacts with mouse IgG1 (reviewed in 1,2). Differences in structure and cellular distribution of the two receptors were demonstrated - inter alia - by mAb with reactivity for either FcγRI (3,4), or FcγRII (5,6). Furthermore, it has recently been shown that the two types of FcγR can be modulated independently from each other (7).

Both FcγR were previously analyzed in detail in T cell proliferation experiments, in which FcγR on monocytes are required to support anti-CD3 induced T cell proliferation. Monocytes from essentially all donors were observed to mediate interactions with mouse (m) IgG2a antibodies (FcγRI-mediated (1,4,8)). Monocytes of some donors (about 30% of Caucasian individuals) were observed to be unable to support anti-CD3 induced T cell proliferation by mIgG1 mAb (low-responder individuals; LR). Monocytes of the other individuals (high-responders; HR) were found to support induction of T cell mitogenesis by mIgG1 mAb effectively (8,9). This polymorphism has been attributed to FcγRII on monocytes (9), is genetically determined, and results from structural differences in FcγRII of HR and LR monocytes (10). Further functional studies analyzing both FcγRI and II have been scarce, although it has been shown that both receptors are involved in superoxide production (3).

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a potentially important immune defense mechanism (11). Until now, most studies have analyzed the function of FcγRI in ADCC with human monocytes as effector cells. Efficient cytotoxicity to erythrocytes coated with different human (12,13) or rabbit (14) antibodies has

been reported, indicating that FcγRI is able to mediate cytotoxicity. This was also suggested by studies of Koprowski and colleagues, who analyzed the cytotoxicity of human monocytes to various tumour targets sensitized with different mouse mAb. These authors generally found that only mIgG2a mAb, and not mAb of other isotypes (e.g. mIgG1, mIgG2b) were effective (15-17). These studies suggested that FcγRII could not function as a trigger molecule in monocyte-mediated cytotoxicity.

We analyzed the cytotoxicity of human monocytes to sensitized erythrocytes, both as a model of cellular cytotoxicity and because of its possible relevance for diseases such as autoimmune haemolytic anemia (12,13). Two types of target cells were used, HRBC sensitized with human IgG (EA-human IgG), or with mouse IgG1 (EA-mouse IgG1), which were previously found to interact with FcγRI or FcγRII, respectively (5,18). Both types of FcγR were found to mediate cytotoxicity but their efficiency was different. Furthermore, we observed differences in FcγRII-mediated cytolysis of targets between HR and LR individuals.

2. MATERIALS AND METHODS

2.1. Monocytes.

White blood cell suspensions were obtained by cytapheresis of healthy volunteers. Monocytes were isolated from these suspensions by Percoll (Pharmacia, Uppsala, Sweden) centrifugation and elutriation as described before (19). Monocyte fractions consisted for at least 90% of monocytes (evaluated in cytocentrifuge preparations after staining for non-specific esterase and with May-Grünwald-Giemsa), and viability was more than 98%, as assessed by trypan blue exclusion. Since monocytes from different individuals show functional variability we considered it advantageous to have fixed and well-defined batches of these cells. The purified monocytes were therefore cryopreserved (20) and used later. Both recovery and viability of cryopreserved monocytes were excellent (> 70% and > 95%, respectively). All experiments presented in this report were repeated at least once using freshly isolated monocytes, which yielded similar results.

2.2. Anti-FcγR monoclonal antibodies.

The receptors involved in the cytotoxicity of monocytes to sensitized erythrocytes were characterized by using different anti-FcγR

monoclonal antibodies. The anti-FcγRI antibody used was mAb 10.1 (4), whereas anti-FcγRII antibodies employed were mAb IV.3 (5) obtained from Clark Anderson, and mAb CIKM5 (6), a kind gift from Glenn Pilkington.

2.3. Ig preparations.

For the characterization of monocyte FcγR involved in ADCC to HRBC, and for quantitation of the number of IgG molecules on the target cells, we used the following Ig preparations. The mouse mAb used were PA1 (IgG1 antibody against idiotypic determinant on B-CLL cells, produced by Frank Preyers, Nijmegen), 3-1-3-1 (IgG2b anti-mouse H-2 determinant)(21), and 2-2-1 (IgG2a anti-mouse Ia determinant)(21). These antibodies were purified from ascites fluid by $(NH_4)_2SO_4$ precipitation, followed by different chromatographic procedures (22). No residual antibodies of a contaminating subclass could be detected by double diffusion in agarose (i.e. more than 99.9% pure). In some experiments we used human IgG, which was purified from human serum by ammonium sulphate precipitation and DEAE-Sephadex (Pharmacia, Uppsala, Sweden) chromatography. The final preparation of human IgG was checked for impurities and IgG content in immunoelectrophoresis and radial immunodiffusion, and appeared 99.9% pure.

After isolation, all fractions were depleted of aggregates by ultracentrifugation (1 h at 100,000 g, 4°C), and the protein concentrations were determined by the method described by Bradford et al. (23) using bovine gamma globulin (Bio-Rad, Munich, F.R.G.) as a standard. The proteins were rapidly snap-frozen in small aliquots in liquid nitrogen, stored at -20°C and thawed once, just before their use in the experiments.

2.4. T cell proliferation assay

The accessory function of monocytes in anti-CD3 induced T cell proliferation was studied for all donors as described before (8). Anti-CD3 mAb used were WT32 (mIgG2a)(24), and UCHT1 (mIgG1) (25), a kind gift of Peter Beverley (London, U.K.).

2.5. Cytotoxicity assay

Human monocytes from different donors were tested for their ADCC capacity in a ^{51}Cr release assay in which antibody-coated human erythrocytes were used as target cells. A quantity of 10^8 HRBC were suspended in 0.3 ml PBS containing 100 μCi ^{51}Cr (sodium chromate, Radiochemical Centre, Amersham, U.K.) and incubated at 37°C. After 30 min, the incubation volume was increased to 1 ml, and an equal volume of sensitizing antibody (or PBS, as a control) was added. Two types of sensitizing antibodies were used. In order to obtain EA-human IgG we used a human antiserum against rhesus D (Merz & Dade, Düdingen, Switzerland) in combination with rhesus D-positive HRBC. A monoclonal anti-glycophorin A antibody (a generous gift of Marjolein Bos, CLB, Amsterdam) was used to construct EA-mouse IgG1. Both types of antibodies were used in various dilutions, re-

sulting in erythrocytes sensitized with a range of IgG molecules per cell. After incubation for 30 min with continuous gentle agitation, cells were washed thrice with PBS containing 0.1% bovine serum albumin (Cohn fraction V, Sigma, St.Louis, Mo., USA), suspended at $2.5x10^6$ cells/ml in RPMI 1640 medium plus 10% FCS, and used immediately. Target cell numbers were calculated by counting unlabelled HRBC, which were included in all experiments for each dilution of sensitizing antiserum.

Various concentrations of effector cells (in RPMI 1640 medium with 10% FCS) were seeded in U-bottomed microtiter plates (Costar, Cambridge, Mass., USA) in a volume of 100 µl/well. Hundred microliters of target cells were supplemented to the wells, plates were centrifuged (2 min, 50 g, room temperature) and incubated for 18 h at 37°C. After incubation, plates were centrifuged (10 min, 400 g, room temperature), 100 µl of supernatant fluid was collected and counted in an LKB gamma counter. The ADCC was expressed as percent cytotoxicity, which was calculated as follows: 100 x (A-B/C-B), where A is mean cpm of test sample; B, mean cpm of spontaneous ^{51}Cr release (i.e. ^{51}Cr release by labelled target cells in medium only); and C, mean cpm of the maximal ^{51}Cr release that was obtained by addition of 20% saponin (Coulter, Dunstable, U.K.) to the target cells. The average spontaneous release ± standard deviation of the HRBC was 3% ± 1%, and release from uncoated target cells incubated with monocytes did not exceed spontaneous release. In all experiments tests were conducted in triplicate and the results expressed as mean ± standard deviation.

2.6. Quantitation of erythrocyte sensitization.
Since ADCC is critically dependent on the degree of target cell sensitization, this variable was studied in more detail. Erythrocyte-bound antibodies were quantitated by their ability to inhibit the binding of standardized amounts of ^{125}I-IgG by anti-IgG antisera. The binding of ^{125}I-IgG was quantitated by precipitation of the immune complexes with 6% PEG, as has recently been described in detail (26).

3. RESULTS

3.1. Cytotoxic activity of monocytes against erythrocytes sensitized with human IgG or mouse IgG1

Monocytes from HR and LR donors were tested for their cytotoxic activity against two types of sensitized HRBC. HR monocytes were cytotoxic to EA-human IgG, as well as to EA-mouse IgG1 (Fig.1).
The target cells were maximally sensitized with antibodies in this experiment, and it can be observed that HR monocytes killed both types of target cells with equal effectiveness. LR monocytes, how-

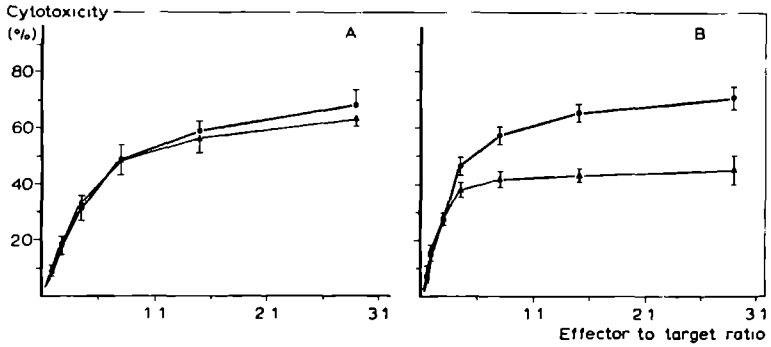

Fig.1. Lysis of erythrocytes, maximally sensitized with human IgG (A) or mouse IgG1 (B), by monocytes from an HR (●) and an LR (▲) individual. The results are representative of similar experiments using monocytes from six LR and seven HR individuals.

ever, were less cytotoxic to EA-mouse IgG1 than HR monocytes, whereas cytotoxicity to EA-human IgG was the same as with HR monocytes. In control experiments it was shown that both unsensitized HRBC, as well as HRBC sensitized with $F(ab')_2$ fragments of the mouse IgG1 mAb (against glycophorin A), were not killed by human monocytes (results not shown). This indicates that the observed effects are Fc-mediated. The differences between monocytes from HR and LR individuals were found for a large number of donors. The maximal level of cytotoxicity to EA-mouse IgG1 was found to be 47.6±5.6% (mean ± SD; n=12) with LR monocytes, and 69.5±1.7% (n=16) with HR monocytes. Maximal cytolysis of EA-human IgG was 68.1±3.3% (n=9) with LR monocytes, and 69.7±4.7% (n=4) with HR monocytes. Furthermore, this pattern was observed to remain constant for individual donors who were followed over a period of 1.5 years.

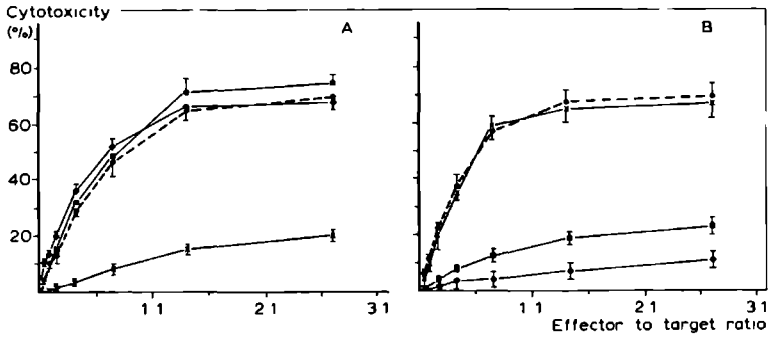

Fig.2. Effect of monoclonal anti-FcγR antibodies on the lysis by HR monocytes of erythrocytes, maximally sensitized with human IgG (A) or mouse IgG1 (B). Monocytes and target cells were incubated either in RPMI 1640 medium plus 10% FCS alone (control) (●; dashed line), or in medium containing either anti-FcγRII mAb IV.3 (culture supernatant, 1:80)(■) or CIKM5 (ascites fluid, 1:100) (◆), or anti-FcγRI mAb 10.1 (acites fluid, 1:1000)(x). Results are representative for four experiments.

3.2. Characterization of monocyte ADCC by different monoclonal anti-FcγR antibodies

Different anti-FcγR mAb were tested for their effect on ADCC mediated by monocytes with EA-human IgG and EA-mouse IgG1 as target cells. Two anti-FcγRII mAb, IV.3, and CIKM5 both inhibited the cytotoxicity of monocytes to EA-mouse IgG1, whereas cytotoxicity to EA-human IgG was not affected (Fig.2). Both antibodies were previously found to inhibit the binding of mouse IgG1 to FcγRII (5,6,18), and react with different epitopes on this FcγR (Dr. Tetteroo, personal communication). Another mAb 10.1, which specifically binds to the Fc binding epitope of FcγRI (4), inhibited the cytotoxicity to EA-human IgG, whereas ADCC to the other type of tar-

get cells was unaffected (Fig.2). These observations indicate that FcγRI mediates the cytotoxicity of monocytes to EA-human IgG, and that FcγRII mediates the kill of EA-mouse IgG1. Similar results were obtained when LR monocytes were used (data not shown).

3.3. Effects of homologous and heterologous IgG on ADCC mediated by FcγRI and FcγRII.

After having defined the structures on monocytes which mediate the kill of the two types of target cells, we investigated the inhibitory effect of monomeric Ig on target cell lysis. Fig.3 shows that monomeric human IgG inhibits in a dose-dependent way the cytotoxicity of monocytes to EA-human IgG, whereas kill of EA-mouse IgG1 was not affected. Similar results were obtained using HR monocytes (data not shown).

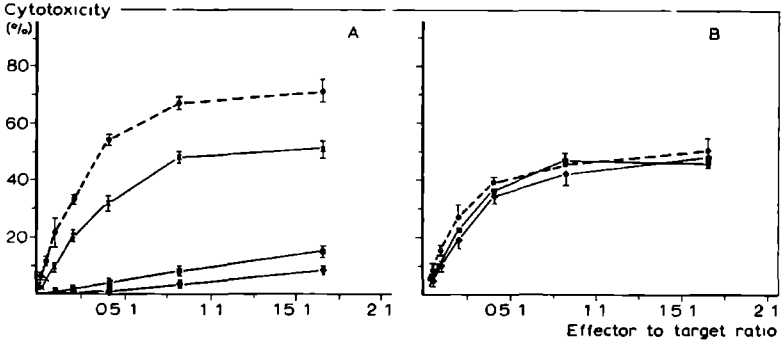

Fig.3. Effect of monomeric human IgG on monocyte-mediated lysis of erythrocytes, maximally sensitized with human IgG (A) or mouse IgG1 (B). Monocytes from an LR individual were incubated with target cells in RPMI 1640 medium with 10% FCS alone (control) (●; dashed line), or in medium containing human IgG at a concentration of 1 μg/ml (x), 10 μg/ml (■), or 25 μg/ml (◆). Results are representative for three experiments.

Mouse immunoglobulins have previously been used frequently to dissect the heterogeneity of human monocyte FcγR (e.g. in 18,27). Fig.4 shows that mIgG2a inhibited the ADCC to EA-human IgG, whereas ADCC to EA-mouse IgG1 remained unaffected. The cytotoxicity of monocytes to EA-mouse IgG1 was observed to be inhibited by mIgG1, in a dose-dependent way. Mouse immunoglobulins of the IgG2b isotype had no influence on the killing of both types of target cells.

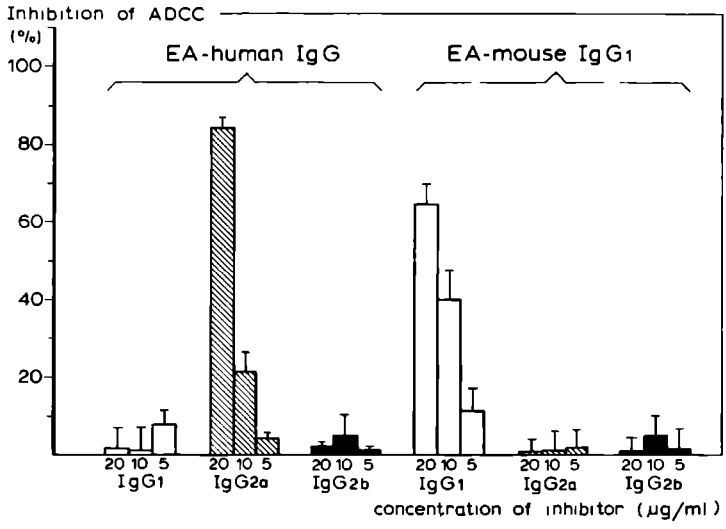

Fig.4. Inhibition of monocyte cytotoxicity to sensitized erythrocytes by monomeric mouse immunoglobulins of different isotypes. The effector to target ratio was 1.5, monocytes were from an HR individual, and target cells were maximally sensitized. The percentage inhibition was calculated as follows:

$$\left[1 - \frac{\%\ \text{Cytotoxicity with inhibitor}}{\%\ \text{Cytotoxicity in medium with 10\% FCS}}\right] \times 100$$

This experiment was repeated four times with comparable results.

3.4. Effect of degree of sensitization on ADCC

Until now target cells were used which were maximally sensitized. We have previously observed that the degree of erythrocyte sensitization obtained with anti-Rhesus D antibody and with anti-glycophorin A mAb shows large differences. Erythrocytes were found to bind maximally ± 20,000 human IgG anti-Rhesus D molecules/HRBC whereas they could be sensitized with as much as 400,000 mouse IgG1 anti-glycophorin A molecules/target cell without agglutination (26). Since the degree of target cell sensitization influences ADCC, we quantitated this parameter in more detail, and studied its effect on monocyte cytotoxicity. Striking differences were observed when EA-human IgG and EA-mouse IgG1 were compared as target cells for cytotoxicity by monocytes (Fig.5). Half-maximal cytotoxicity of monocytes to EA-human IgG was observed with targets containing approximately 5,500 IgG molecules, whereas for half-maximal cytolysis

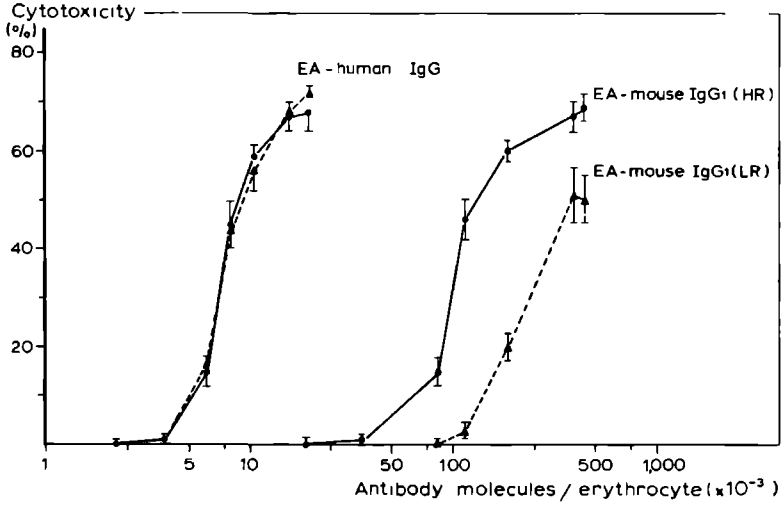

Fig.5. Effect of ligand density on ADCC of human monocytes to two types of sensitized erythrocytes. Cytotoxic activity was studied with monocytes from individuals of both the HR (●-●) and LR (▲---▲) phenotype. Effector to target ratio was 1.8.

of EA-mouse IgG1, approximately 100,000 IgG molecules per HRBC were needed when HR monocytes were used (Table 1). In contrast, approximately 200,000 molecules of mouse IgG1 per HRBC were needed for half-maximal lysis with LR monocytes. Furthermore, as described in section 3.1, the maximal level of monocyte-mediated lysis of EA-mouse IgG1 was always lower than that of HR monocytes.

3.5. Mechanisms involved in ADCC of erythrocytes

Direct cytolysis, but not phagocytosis, is known to play the most important role in monocyte-mediated cytotoxicity to HRBC, sensitized with anti-rhesus D antibodies (12). We investigated whether the same is true for cytotoxicity against EA-mouse IgG1. We therefore studied ADCC of monocytes to the two types of target cells both in the presence and absence of 0.15 mM iodoacetate (a concentration which completely inhibits phagocytosis)(28). Cytolysis of EA-human IgG was not affected at all, whereas lysis of EA-mouse IgG1 was only inhibited by 4.6±1.3% (mean ± SD, 2 experiments). We next investigated the possible contribution of tumour necrosis factor (TNF) to cytolysis of sensitized erythrocytes by performing ADCC experiments in the presence of neutralizing anti-TNF antiserum, which yielded similar results as those in the absence of antiserum (data not shown).

Table 1. Cytotoxic activity of monocytes from HR and LR individuals against two types of sensitized erythrocytes

Target cells	Target cell sensitization (IgG molecules/cell x 10^{-3}) at half-maximal cytotoxicity with		P
	High Responder Monocytes	Low Responder Monocytes	
EA-human IgG	5.3 ± 2.2 (8)[a]	5.8 ± 2.0 (10)	NS[b]
EA-mouse IgG1	95.5 ± 45.8 (8)	206 ± 67.9 (10)	P<0.003

a. Values represent means ± standard deviation, with the number of experiments performed in parentheses.
b. Statistical analysis: Wilcoxon two-sample test

4. DISCUSSION

Human monocytes express two types of receptors for the Fc moiety of IgG, FcγRI and FcγRII (1,2). These receptors can be modulated selectively by immobilized immune complexes, which indicates that they represent independent membrane structures (7). Most studies of antibody dependent cell-mediated cytotoxicity (ADCC) by monocytes have focused on the role of FcγRI. In the present study we have compared the cytotoxic activity mediated by FcγRI and by FcγRII. Human erythrocytes sensitized with either human IgG (EA-human IgG) or with mouse IgG1 (EA-mouse IgG1) were used as target cells. When sensitization was maximal, both types of target cells were lysed at a similar effector to target ratio. Monoclonal anti-FcγR antibodies as well as purified (monomeric) immunoglobulins were used to investigate which type of FcγR was involved. Anti-FcγRI mAb 10.1 (4) was found to inhibit cytolysis of EA-human IgG but not of EA-mouse IgG1. Lysis of EA-human IgG (but not EA-mouse IgG1) was also inhibited by human IgG and by mouse IgG2a. FcγRI therefore appears to be responsible for monocyte ADCC towards EA-human IgG. Two mAb against FcγRII, IV.3 (5) and CIKM5 (6), as well as monomeric mouse IgG1 were able to inhibit cytolysis of EA-mouse IgG1. These findings indicate that FcγRII is involved in lysis of mouse IgG1-sensitized target cells. Recently, Graziano and Fanger (29) demonstrated that hybridoma cells IV.3, which bear the antibody directed to FcγRII, can be lysed by human monocytes, which also indicates that FcγRII can function as a trigger molecule in ADCC.

It is well known that FcγRII is a polymorphic structure. This structural polymorphism is reflected in polymorphism of the mitogenic effect of mouse IgG1 anti-CD3 antibodies (8,9). Monocytes from "low-responder"(LR) individuals can interact only weakly with mouse IgG1, as measured in rosette assays (18). The two different forms of FcγRII are both recognized by mAb IV.3, but have different isoelectric focussing patterns (10). Although we found that both LR and HR monocytes were able to lyse EA-mouse IgG1, the percentage of

specific lysis was consistently higher for HR monocytes than for LR monocytes. Similarly, Graziano and Fanger (29) observed that both HR and LR monocytes could kill hybridoma cells bearing anti-FcγRII antibody, bot only the HR monocytes could in addition also kill hybridoma cells bearing mouse IgG1 with "irrelevant" specificity.

An important parameter in ADCC is the degree of target cell sensitization (17). Cytotoxicity mediated by FcγRI was found to be much more efficient than that mediated by FcγRII. Approximately 20 times more IgG molecules were needed per target cell for half-maximal lysis mediated by this last receptor when HR monocytes were tested. When LR monocytes were used, approximately twice as many mIgG1 molecules/HRBC were needed to get half-maximal lysis, compared with HR monocytes. The lower efficiency of ligand-binding by FcγRII relative to FcγRI, as well as the difference between HR and LR monocytes have previously also been observed when analyzing EA-rosetting between monocytes and HRBC sensitized with human IgG or mouse IgG1 (26). At least 30 times more mouse IgG1 than human IgG molecules had to be present per HRBC for half-maximal rosetting with monocytes from HR individuals. With LR monocytes this number was twice as high as for HR monocytes.

The significance for immunotherapy of differences in cytotoxic efficiency between FcγRI and FcγRII is illustrated by experiments reported by Steplewski et al. (16). These authors showed that a mIgG1 anti-tumour mAb was not able to induce cytolysis with monocytes as effector cells, but the mIgG2a isotype-switch variant of the same antibody was perfectly able to induce kill of tumour cells, both in vitro and in vivo (in nude mice). The physiological significance of FcγRI, however, has been questioned before (4), since this receptor has a high affinity for monomeric human IgG (Ka 10^8-10^9 M^{-1})(1,6) and is likely to be saturated with human IgG which is present at approximately 10^{-5}M in serum. FcγRII on the other hand will not, or to a much lesser extent, be blocked by human IgG (see for instance Fig.3).

Monocyte-mediated cytotoxicity of sensitized HRBC did not depend on phagocytosis, as indicated by experiments performed in the presence of the phagocytosis-inhibitor iodoacetate (28) which yielded similar results as those in the absence of this substance. It has been shown, previously, that FcγRI-mediated cytolysis is not dependent on phagocytosis and can be attributed to the release of lysosomal enzymes by monocytes (13). Reactive oxygen species did not play an important role in cytotoxicity of monocytes to sensitized HRBC (30). It was recently found that tumour necrosis factor (TNF) plays a role in monocyte-mediated cytotoxicity against tumour cells (31). When we investigated the contribution of this molecule to cytolysis of sensitized erythrocytes, no effect could be observed. We therefore conclude, that both types of FcγR mediate kill of sensitized erythrocytes, not by phagocytosis, but by a cytolytic mechanism which may involve the secretion of lysosomal enzymes by monocytes (13).

In conclusion, we found that not only FcγRI but also FcγRII on human monocytes is functional in ADCC to erythrocytes. Furthermore, the cytotoxicity mediated by FcγRII was polymorphic, in concordance with the results found previously in anti-CD3 induced T cell proliferation (8,9) and in rosette assays (18). The degree of sensitization of target cells required for half-maximal cytotoxicity is much higher for FcγRII-mediated lysis, and in this respect the efficacy of killing by FcγRII is much lower than for FcγRI.

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5. REFERENCES

1. Anderson, C.L. and Looney, R.J., Immunol.Today 1986. 7: 264.
2. Morgan, E.L. and Weigle, W.O., Adv.Immunol. 1987. 40: 61.
3. Anderson, C.L., Guyre, P.M., Whitin, J.C., Ryan, D.H., Looney, R.J. and Fanger, M.W., J.Biol.Chem. 1986. 261: 12856.
4. Dougherty, G.J., Selvendran, Y., Murdoch, S., Palmer, D.G. and Hogg, N., Eur.J.Immunol. 1987. 17: 1453.
5. Looney, R.J., Abraham, G.N. and Anderson, C.L., J.Immunol. 1986. 136: 1641.
6. Tetteroo, P.A.T., Van der Schoot, C.E., Visser, F.J., Bos, M.J.E. and Von dem Borne, A.E.G.Kr., in McMichael, A.J. et al. Eds.), Leucocyte Typing III, Oxford University Press, Oxford 1987, p. 702.
7. Van de Winkel, J.G.J., Van Duijnhoven, J.l.P., Van Ommen, R., Capel, P.J.A. and Tax, W.J.M., J.Immunol. 1988. 140: 3515.
8. Tax, W.J.M., Willems, H.W., Reekers, P.P.M., Capel, P.J.A. and Koene, R.A.P., Nature 1983. 304: 445.
9. Tax, W.J.M., Hermes, F.F.M., Willems, H.W., Capel, P.J.A. and Koene, R.A.P., J.Immunol. 1984. 133: 1185.
10. Anderson, C.L., Ryan, D.H., Looney, R.J. and Leary, P.C., J.Immunol. 1987. 138: 2254.
11. Shaw, G.M., Aminoff, D., Balcerzak, S.P. and LoBuglio, A.F., J.Immunol. 1980. 125: 501.
12. Kurlander, R.J., Rosse, W.F. and Logue, G.L., J.Clin.Invest. 1978. 61: 1309.
13. Fleer, A., Van Schaik, M.L.J., Von dem Borne, A.E.G.Kr. and Engelfriet, C.P., Scand.J.Immunol. 1978. 8: 515.
14. Shen, L., Guyre, P.M., Anderson, C.L. and Fanger, M.W., J.Immunol. 1986. 137: 3378.
15. Steplewski, Z., Lubeck, M.D. and Koprowski, H., Science 1983. 221: 865.
16. Steplewski, Z., Spira, G., Blaszczyk, M., Lubeck, M.D., Radbruch, A., Illges, H., Herlyn, D., Rajewsky, K. and Scharff, M., Proc.Natl.Acad.Sci.USA 1985. 82: 8653.
17. Herlyn, D., Powe, J., Ross, A.H., Herlyn, M. and Koprowski, H., J.Immunol. 1985. 134: 1300.
18. Van de Winkel, J.G.J., Tax, W.J.M., Van Bruggen, M.C.J., Van Roozendaal, C.E.P., Willems, H.W., Vlug, A., Capel, P.J.A. and Koene, R.A.P., Scand.J.Immunol. 1987. 26: 663.
19. Van de Winkel, J.G.J., Tax, W.J.M., Van Bruggen, M.C.J., Van Roozendaal, C.E.P., Willems, H.W., Van Duijnhoven, J.L.P., Capel, P.J.A. and Koene, R.A.P., J.Immunol.Methods 1987. 101: 109.
20. Shah, V.O., McCarley, D.L. and Weiner, R.S., Cryobiology 1984. 21: 475.
21. Lems, S.P.M., Tax, W.J.M, Capel, P.J.A. and Koene, R.A.P., J.Immunol. 1983. 131: 86.
22. Lems, S.P.M., Rijke-Schilder, T.P.M., Capel, P.J.A. and Koene R.A.P., Transplantation 1984. 37: 291.
23. Bradford, M.M. Anal.Biochem. 1976. 72: 248.

24. Tax, W.J.M., Leeuwenberg, H.F.M., Willems, H.W., Capel, P.J.A. and Koene, R.A.P., in Bernard, A. et al. (Eds.), Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies, Springer-Verlag, Berlin 1984, p. 721.
25. Beverley, P.C.L. and Callard, R.E., Eur.J.Immunol. 1981. 11: 329.
26. Van de Winkel, J.G.J., Tax, W.J.M., Groeneveld, A., Tamboer, W.P.M., De Mulder, P.H.M. and Capel, P.J.A., J.Immunol.Methods 1988. 108: 95.
27. Jones, D.H., Looney, R.J. and Anderson, C.L., J.Immunol. 1985. 135: 3348.
28. Walker, W.S. and Demus, A., J.Immunol. 1975. 114: 765.
29. Graziano, R.F. and Fanger, M.W., J.Immunol. 1987. 139: 3536.
30. Fleer, A., Roos, D., Von dem Borne, A.E.G.Kr. and Engelfriet, C.P., Blood 1979. 54: 407.
31. Nissen-Meyer, J., Austgulen, R. and Espevik, T., Cancer Res. 1987. 47: 2251.

CHAPTER 7

FcγRII ON "LOW-RESPONDER" HUMAN MONOCYTES ACQUIRES HIGH AFFINITY FOR MOUSE IgG1 BY PROTEOLYSIS

Jan G.J. van de Winkel, Patricia J.T.A. Groenen,
Truus P.M. Rijke-Schilder, René van Ommen, and Wil J.M. Tax

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Two types of IgG Fc receptors, FcγRI and FcγRII, are present on human monocytes. They can be assayed by EA-rosetting, using erythrocytes sensitized with human IgG or mouse IgG1, respectively. On mouse macrophages, FcγRI is sensitive to trypsin while FcγRII is trypsin-resistant. We have now studied the effects of the proteolytic enzymes pronase and trypsin on human monocyte Fcγ receptors. Trypsin nor pronase caused a decrease in rosetting mediated by human monocyte FcγRI. Human monocyte FcγRII is polymorphic, as has been demonstrated previously in studies on T cell proliferation induced by mouse IgG1 anti-CD3 antibodies. Monocytes are required for this proliferation, and individuals can either be high-responder or low-responder depending on the strength of the interaction of monocyte FcγRII with mouse IgG1. We observed that the interaction of low-responder monocytes with mouse IgG1 was dramatically increased (to the level exhibited by high-responder monocytes) by treating the monocytes with proteolytic enzymes. Since the number of Fc receptors was not affected (as measured by immunofluorescence using anti-FcγR monoclonal antibodies), we conclude that the affinity for ligand was increased by proteolysis. At sub-optimal sensitization of indicator erythrocytes, the enhancing effect of protease-treatment could also be demonstrated with high-responder monocytes, both in EA-rosetting and in antibody-dependent cytotoxicity.

1. INTRODUCTION

Human monocytes constitutively express two types of receptors for the Fc moiety of IgG: FcγRI and FcγRII. FcγRI has a high affinity for both monomeric and complexed human IgG, while FcγRII has only a low affinity for complexed human IgG. This latter receptor cross-reacts with mouse IgG1, and is structurally polymorphic (reviewed in 1,2). Monocytes from different donors interact either strongly or weakly with mouse IgG1 (high-, respectively low-responder individuals; HR or LR), as observed in studies on T cell proliferation induced by mouse IgG1 anti-CD3 antibodies (3) and in rosetting experiments with mouse IgG1-sensitized erythrocytes (4,5).

Knowledge of the protease sensitivity of human monocyte FcγR is fragmentary. FcγRI of human monocytes was reported to be resistant to both trypsin (6) and pronase (7), whereas the effects on FcγRII have not been described before. The different types of FcγR present on mouse macrophages exhibit a differential sensitivity to trypsin (reviewed in 8). Since the data on the effects of proteases on human monocyte FcγR are incomplete we carried out a detailed study of the effects of trypsin and pronase on FcγRI and FcγRII of human monocytes.

2. MATERIALS AND METHODS

2.1. Monocytes

Cytapheresis of healthy volunteers was applied to obtain white blood cell suspensions. Monocytes were isolated from these suspensions by Percoll (Pharmacia, Uppsala, Sweden) centrifugation and counterflow centrifugation as described before (9). Monocyte suspensions consisted for at least 90% of monocytes (evaluated in cytocentrifuge preparations after staining for non-specific esterase and with May-Grünwald-Giemsa) and viability was higher than 98% (assessed by trypan blue exclusion). Isolated cells were either used immediately or were cryopreserved (10) and stored in liquid nitrogen until use. Most experiments were performed with cryopreserved monocytes, but all types of experiments were performed at least once using freshly isolated cells, which yielded similar results.

2.2. T cell proliferation assay

The accessory function of monocytes in anti-CD3 induced T cell proliferation was studied for all donors as described before (3). Anti-CD3 mAb used were WT32 (mIgG2a) (11), and UCHT1 (mIgG1) (12), a kind gift of Dr. Beverley (London, U.K.).

2.3. Anti-FcγR monoclonal antibodies

Anti-FcγRI monoclonal antibodies used were 10.1 provided by Dr. Hogg (13), and 32.2 from Dr. Anderson (2). Two mAb reactive with FcγRII were used; IV.3 a gift from Dr. Anderson (14), and CIKM5 provided by Dr. Pilkington (15). The following anti-FcγRIII mAb were employed: anti-Leu 11a (16), and anti-Leu 11b (17), both from Becton Dickinson (Mountain View, Ca.).

2.4. Treatment with proteolytic enzymes

Monocytes were incubated in suspension (at a concentration of $4x10^6$ cells/ml) with RPMI 1640 medium alone (control) or with medium containing either 0.5 mg/ml trypsin, or 0.2 mg/ml pronase (both from Boehringer, Mannheim, FRG), for 30 min at 37°C. After three washes, cells were resuspended in RPMI 1640 medium containing 5% FCS and rosette formation was assayed as described below. Losses of cells after treatment with proteases were small and amounted to 6.0±4.9% (n=4; freshly isolated monocytes), and 15.5±10.3% (n=16; cryopreserved monocytes), relative to cells incubated in medium alone. Viability of cells after proteolysis was always higher than 95%.

In some experiments monocyte-monolayers were prepared in microtitre plates as described before (9). Briefly, 100 µl of a cell suspension ($4x10^6$ cells/ml in RPMI 1640 with 10% FCS) were dispensed into individual wells of flat-bottomed microtitre plates (Costar, Cambridge, MA) and incubated for 1 h at 37°C. After two washes, wells were supplemented with RPMI 1640 medium alone (control), or with medium containing either 0.5 mg/ml trypsin or 0.2 mg/ml pronase and plates were incubated for 30 min at 37°C. After two washes, sensitized erythrocytes were added and binding was quantitated as described below. Viability of monocytes was high (> 95%, assessed by trypan blue exclusion) and was not decreased by protease treatment, nor was the number of cells per well influenced as evaluated by microscopical examination.

2.5. Immunofluorescence

The expression of different monocyte antigens on enzyme-treated cells was assayed by indirect immunofluorescence, using various mAb and a fluorescein-conjugated $F(ab')_2$ fraction of sheep anti-mouse IgG antiserum (Cappel, Malvern, Pa.). Complement receptor type 3 was evaluated by using mAb OKM1 (18) from Becton Dickinson (Mountain View, Ca.), monocyte/macrophage specific antigen p55 (CD14) by mAb Mo2 (19) from Coulter Immunology (Hialeah, Fl.), and the different types of FcγR by the mAb described in section 2.3. Fluorescence was determined with an Ortho 50-H flow cytometer. In each sample the fluorescence intensity from 10,000 cells was measured.

2.6 EA-rosetting

Two types of indicator cells were used for EA-rosette assays. Rhesus D-positive human erythrocytes (HRBC) were sensitized either with a human alloserum against rhesus D (Merz & Dade, Düdingen, Switzerland), or with a mouse IgG1 mAb against glycophorin A (obtained from M. Bos, CLB, Amsterdam). In some experiments, HRBC were sensitized with $F(ab')_2$ fragments of the mIgG1 anti-glycophorin A mAb. Preparation of $F(ab')_2$ fragments and sensitization of HRBC were performed as described before (9).

Rosetting was studied using two different methods, a microscopic and a photometric one. The microscopic assay was performed essentially as described before (9). Briefly, to 200 µL of cells (at $2x10^6$/ml in RPMI 1640 with 5% FCS) 50 µl of sensitized HRBC suspension (0.5% v/v) were added, the samples were centrifuged 4 min at 20 g, and incubated for 1 h at 4°C. Cells with at least three bound HRBC were scored as rosettes, using a microscope with 400 x final magnification. Unsensitized HRBC were included in each experiment, and never found to form rosettes with any of the cells tested (neither protease-treated, nor untreated). At least 200 cells were scored per incubation.

In inhibition experiments anti-FcγR mAb were added to monocytes after incubation with proteases, and experiments were further performed under similar conditions as described above.

Binding of sensitized HRBC (and non-sensitized HRBC as a control for non-specific binding) to monocyte-monolayers was evaluated after incubation for 1 h at 4°C. Quantitation of binding was performed photometrically at 450 nm using the pseudoperoxidase activity of HRBC as indicator (9). Binding indices were calculated, in order to correct for variations in background absorbance values between different experiments, as follows:
$[1 - (E450/EA450)] \times 100$ = binding index, where E450 is extinction at 450 nm in wells to which HRBC without sensitizing antibody were added, and EA450 is extinction at 450 nm in wells to which sensitized HRBC were added. All determinations were performed in duplicate, and the variability was less than 10%.

2.7. Cytotoxicity assay

Monocytes were tested for their ADCC capacity in a ^{51}Cr release assay in which antibody-coated HRBC were used as targets. 10^8 HRBC were suspended in 0.3 ml PBS containing 100 µCi ^{51}Cr (sodium chromate, Radiochemical Centre, Amersham, U.K.) and incubated at 37°C. After 30 min, the incubation volume was increased to 1 ml, and an equal volume of sensitizing antibody (or PBS, as a control) was added. After incubation for 30 min at 37°C with continuous gentle agitation, cells were washed thrice with PBS containing 0.1% bovine serum albumin (Cohn fraction V, Sigma, St.Louis, Mo.), suspended at $2.5x10^6$ HRBC/ml in RPMI 1640 medium plus 10% FCS, and used immediately. Target cell numbers were calculated by counting unlabelled HRBC, which were included in all experiments.

Various concentrations of effector cells (protease-treated or, as a control, untreated), suspended in RPMI 1640 medium with 10% FCS, were seeded in U-bottomed microtitre plates (Costar, Cambridge, Ma.) in a volume of 100 µl/well. Hundred microliters of target cells were supplemented to the wells, plates were centrifuged (2 min, 50 g) and incubated for 18 h at 37°C. After incubation, plates were centrifuged (10 min, 400 g), 100 µl of supernatant fluid was collected and counted in a LKB gamma counter. The ADCC was expressed as percent cytotoxicity, calculated as follows: 100 x (A-B/C-B), where A is mean cpm of test sample; B, mean cpm of spontaneous ^{51}Cr release (i.e. ^{51}Cr release by labelled target cells in medium only); and C, mean cpm of the maximal ^{51}Cr release that was obtained by addition of 20% saponin (Coulter, Dunstable, U.K.) to the target cells. The average spontaneous release ± SD of HRBC was 3% ± 1%, and release from unsensitized HRBC incubated with monocytes did not exceed spontaneous release.

2.8. Statistics

The Wilcoxon two-sample test was used to evaluate the statistical significance of differences in EA-rosette formation between protease-treated and untreated cells.

3. RESULTS AND DISCUSSION

We have studied the effects of proteases by examining the rosetting mediated by FcγRI and FcγII on human monocytes. The Fcγ receptors

Table 1. Effect of Trypsin and Pronase on EA-rosetting between high-responder monocytes and two types of optimally sensitized erythrocytes

	Binding of sensitized erythrocytes			
	EA-human IgG		EA-mouse IgG1	
Incubation with	% Rosettes	Binding relative to Control (%)	% Rosettes	Binding relative to Control (%)
Buffer	79.2±7.5 (5)[a]	100	70.5±8.2 (4)	100
Trypsin	77.9±4.4 (5)	98	78.9±7.6 (4)	112
Pronase	76.2±10.5(4)	96	84.4±7.3 (4)	120

a. Data were obtained with monocytes from three different donors, and results represent the mean ± SD, with number of individual experiments in parentheses

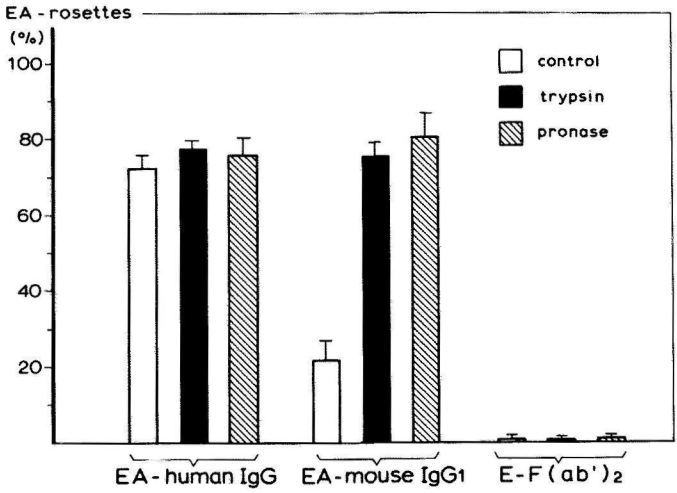

Fig.1. Effect of trypsin and pronase on EA-rosetting between low-responder monocytes and erythrocytes sensitized with either human IgG, mouse IgG1, or $F(ab')_2$ fragments of mouse IgG1. Monocytes were incubated in RPMI 1640 medium alone (control) or in medium containing either trypsin or pronase, washed, and rosetting with maximally sensitized erythrocytes was scored microscopically. Rosetting was performed with monocytes from four donors, and results are expressed as means ± SD of either three (EA-human IgG), nine (EA-mouse IgG1), or two ($E-F(ab')_2$) individual experiments.

were assayed by using erythrocytes sensitized either with human IgG (EA-human IgG; FcγRI) or with mouse IgG1 (EA-mouse IgG1; FcγRII) (5,14). When monocytes from high-responder individuals were used, neither trypsin, nor pronase had any significant effect on rosette formation with optimally sensitized indicator HRBC (Table 1).

Monocytes from about 30% of Caucasian individuals (LR) interact much more weakly with EA-mouse IgG1 than monocytes from the other (HR) donors (4,5), although FcγRII are present in similar amounts (14). When LR monocytes were tested, the degree of EA-human IgG rosetting was similar to that of HR monocytes (±70% rosettes), but

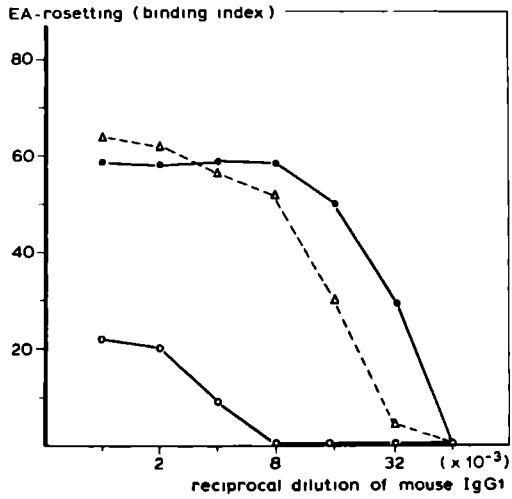

Fig.2. Effect of trypsin on rosette formation between low-responder monocytes and erythrocytes, sensitized at different dilutions of mouse IgG1 anti-glycophorin A antibody. LR monocytes were incubated with medium only (o-o), or with medium containing trypsin (•-•). After washing, the binding of sensitized erythrocytes was assayed photometrically. For comparison, the results obtained with untreated high-responder monocytes (Δ-Δ) are also depicted, results are representative for four experiments.

only about 20% rosettes were formed with EA-mouse IgG1 (Fig.1). Similarly to the results obtained with HR monocytes, no significant effects of the proteases were found on FcγRI-mediated rosette formation. Surprisingly, a greatly enhanced rosette formation with EA-mouse IgG1 (FcγRII-mediated) was seen when LR cells were treated with trypsin ($p<0.003$) or with pronase ($p<0.0002$). This effect was obtained with both freshly isolated and cryopreserved monocytes from all LR donors we tested. The Fc-dependency of EA-rosetting with protease-treated monocytes was demonstrated in a control experiment. Human erythrocytes were optimally sensitized with $F(ab')_2$ fragments of anti-glycophorin A mouse IgG1 antibody (as we used before; 9), and rosette formation with protease-treated (and untreat-

ed) monocytes was studied. We never observed any rosette formation using $F(ab')_2$-sensitized HRBC, neither with untreated, nor with protease-treated monocytes (Fig.1). The experiments described above were carried out with erythrocytes that were maximally sensitized with mouse IgG1. Subsequently, we studied the effects of proteases on EA-mouse IgG1 rosetting of LR monocytes in some more detail, using HRBC sensitized with a range of mouse IgG1 molecules per cell. Erythrocytes were sensitized at different dilutions of mouse IgG1 anti-glycophorin A mAb, resulting in sensitizations varying from about 10,000 to 400,000 mouse IgG1 molecules/HRBC (20). A dramatic enhancement of EA-mouse IgG1 rosette formation was observed when LR monocytes were treated with trypsin (Fig.2), and similar results were obtained with pronase (data not shown). The degree of EA-mouse IgG1 rosetting of LR monocytes after trypsin treatment was found to be comparable to that of untreated HR monocytes (Fig.2). EA-mouse IgG1 rosetting of HR monocytes was also enhanced after protease treatment when we used sub-optimally sensitized indicator HRBC (Table 2).

Table 2. Effect of trypsin on rosetting between HR monocytes and EA-mouse IgG1[a]

Dilution of sensitizing mouse IgG1 antibody ($\times 10^{-3}$)	Expt. 1		Expt. 2	
	control	trypsin	control	trypsin
1:4	66±4[b]	72±7	52±3	68±7
1:8	41±4	68±6	45±5	59±4
1:16	32±2	58±4	10±2	40±3

a. Monocyte-monolayers in microtitre wells were incubated with RPMI 1640 medium alone (control), or with medium containing trypsin for 30 min at 37°C. After washing, binding of indicator erythrocytes, sensitized at different dilutions of mouse IgG1 anti-glycophorin A, was quantitated photometrically.

b. Results represent binding indices calculated as described in Materials and Methods, and are expressed as means ± SD.

We next tested whether this enhanced EA-rosette formation with mouse IgG1-sensitized HRBC by LR monocytes was mediated by FcγRII. Anti-FcγRII mAb IV.3 was found to inhibit the EA-mouse IgG1 rosetting with LR cells after protease treatment by 84.5±5.5% (n=2), whereas anti-FcγRI mAb 10.1 only caused an inhibition of 10.2±3.4% (n=2). Monoclonal antibody IV.3 has previously been shown to block only rosetting between monocytes and EA-mouse IgG1 and not rosette formation with EA-human IgG (5,14). Furthermore, it has been demonstrated previously that anti-FcγRI mAb 10.1 completely inhibits rosette formation between EA-human IgG and human monocytes (13).

We next studied the antibody-dependent cell-mediated cytotoxicity (ADCC) of monocytes towards sensitized HRBC. When HR monocytes were treated with pronase, their ADCC activity to sub-optimally sensitized EA-mouse IgG1 was found to increase drastically (Fig.3). Similar data were obtained when LR monocytes were used (Table 3), and also after treatment with trypsin (data not shown).

Table 3. Effect of pronase on cytotoxicity of LR monocytes to erythrocytes, suboptimally sensitized with mouse IgG1[a]

Effector to target ratio	% cytotoxicity	
	control	pronase
0.25:1	5.3±1.2[b]	7.5±0.6
0.5:1	7.2±1.4	15.0±1.4
1:1	11.0±2.0	25.3±1.9
2:1	13.0±1.0	35.2±3.8

a. Monocytes were incubated in RPMI 1640 medium alone (control) or in medium supplemented with pronase for 30 min at 37°C. After washing, the cells were incubated with ^{51}Cr labelled HRBC (sensitized with ± 186,000 molecules of mouse IgG1 per erythrocyte) at different effector to target ratio's for 18 h and ^{51}Cr release was determined.

b. Means ± SD of triplicate determinations in a representative experiment.

The effect of proteases on the expression of FcγR by monocytes was studied using immunofluorescence analysis. FcγRI was evaluated by using mAb 32.2 and 10.1, directed against different epitopes on the receptor, FcγRII by mAb IV.3 and CIKM5, also reacting with different determinants (Dr. Tetteroo, personal communication). Proteolysis either had no effect, or slightly reduced the expression of FcγRI and FcγRII on monocytes (Fig.4). A third type of FcγR, FcγRIII (CD16) has been described on human granulocytes, macrophages and null cells (2) but not on monocytes. Using two anti-CD16 mAb, anti-Leu 11a and anti-Leu 11b, no induction of this receptor was observed after protease treatment of monocytes. Enzymatic activity of the proteases was confirmed by studying the binding of mAb

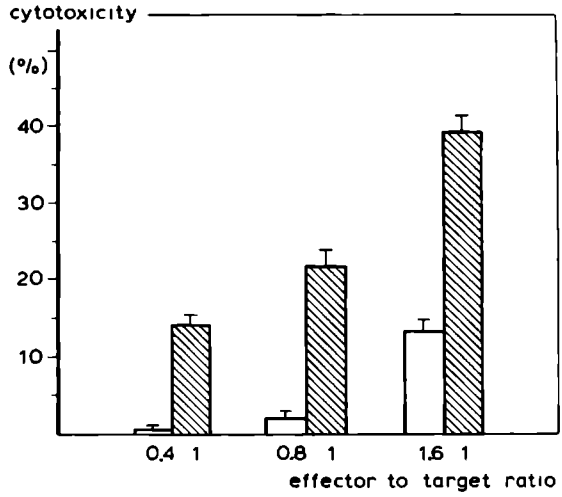

Fig.3. Effect of pronase on cytotoxicity of HR monocytes to erythrocytes, sub-optimally sensitized with mouse IgG1. High-responder monocytes were incubated in the absence (□) or presence (▨) of pronase, washed and incubated with ^{51}Cr labelled HRBC (sensitized with ± 36,000 molecules of mouse IgG1 per erythrocyte) at three different effector to target ratio's for 18 h, after which ^{51}Cr release was determined. Results show the mean ± SD of triplicate determinations in one experiment, which was repeated thrice with comparable results.

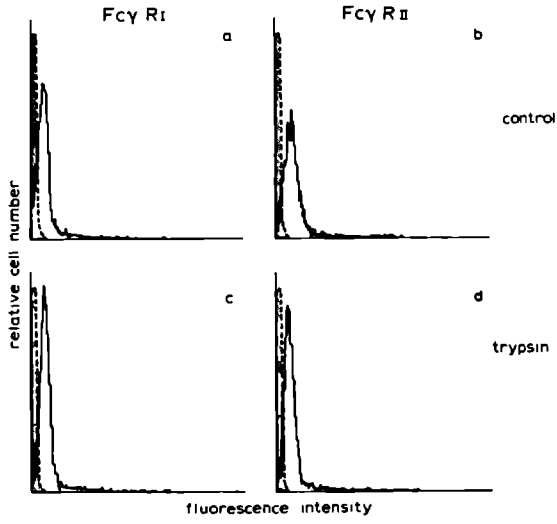

Fig.4. Immunofluorescence analysis of FcγRI and FcγII on monocytes after trypsin treatment. Monocytes were incubated in RPMI 1640 medium alone (Control; a,b) or in medium containing trypsin (c,d). After washing, the expression of FcγRI and II was evaluated with a flow cytometer by using mAb 10.1 (a,c) and IV.3 (b,d), respectively. Fluorescence was recorded as arbitrary units on a linear scale, using RPMI 1640 medium containing 10% FCS alone as a control for non-specific binding (dashed-line). This experiment was repeated twice, employing both trypsin and pronase (data not shown) with comparable results.

to antigens of known proteolytic sensitivity. After protease treatment, binding of mAb Mo2 (which binds to the protease-sensitive CD14 antigen (19)) was reduced by 98% (mean of two experiments) by trypsinization, and by 99% after treatment with pronase. Binding of mAb OKM1, which is known to be protease-resistant (19), was not reduced by proteolysis.

Pronase has previously been found to decrease the interaction of anti-FcγRIII mAb B73.1 with human lymphocytes (21), while trypsin selectively affected mouse macrophage FcγRI (reactive with mIgG2a), but not mouse macrophage FcγRII (reactive with mIgG1/2b),or

mouse FcγRIII (for mIgG3)(8). We found no inhibition of human monocyte FcγRI by these proteases,whereas FcγRII was dramatically increased.

4. CONCLUDING REMARKS

In agreement with previous reports (6,7,22,23), we found that human monocyte FcγRI was not inhibited by the proteases trypsin and pronase. In this regard, human and mouse monocyte FcγR, which are generally assumed to be homologous, appear to differ from each other. The most remarkable result of our study was that with human LR monocytes both EA-mouse IgG1 rosetting and ADCC against mouse IgG1-sensitized HRBC (both mediated by FcγRII) were found to be enhanced considerably by proteolysis. Surprisingly, after protease treatment LR monocytes exhibited a similar degree of EA-mouse IgG1 rosetting as did (untreated) HR monocytes. When sub-optimally sensitized indicator HRBC were used, a protease-induced increase of both EA-mouse IgG1 rosetting and ADCC against mouse IgG1-sensitized HRBC could also be demonstrated with HR monocytes. As the expression of monocyte FcγR does not increase, it is likely that the affinity for IgG is enhanced by limited proteolysis. This is consistent with studies of Cosio et al. (7,23), who reported that trypsin or pronase treatment induces an increase in affinity of human monocytes for rabbit IgG immune complexes, without affecting the number of binding sites for rabbit IgG. This enzyme-mediated modulation of FcγR interactions with ligands may play an important role in vivo at places with increased concentrations of proteases, e.g. at inflammatory sites (24).

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5. REFERENCES

1. Burton, D.R. Molec.Immun. 1985. 22: 161.
2. Anderson, C.L. and Looney, R.J. Immunol.Today 1986. 7: 264.
3. Tax, W.J.M., Willems, H.W., Reekers, P.P.M., Capel, P.J.A. and Koene, R.A.P. Nature 1983. 304: 445.
4. Tax, W.J.M., Hermes, F.F.M., Willems, H.W., Capel, P.J.A. and Koene, R.A.P. J.Immunol. 1984. 133: 1185.
5. Van de Winkel, J.G.J., Tax, W.J.M., Van Bruggen, M.C.J., Van Roozendaal, C.E.P., Willems, H.W., Vlug, A., Capel, P.J.A. and Koene, R.A.P. Scand.J.Immunol. 1987. 26: 663.
6. LoBuglio, A.F., Cotran, R.S. and Jandl, J.H. Science 1967. 158: 1582.
7. Cosio, F.G., Douglas, S.D. and Michael, A.F. Immunology 1982. 46: 395.
8. Unkeless, J.C., Fleit, H. and Mellman, I.S. Adv.Immunol. 1981. 31: 247.
9. Van de Winkel, J.G.J., Tax, W.J.M., Van Bruggen, M.C.J., Van Roozendaal, C.E.P., Willems, H.W., Van Duijnhoven, J.L.P., Capel, P.J.A. and Koene, R.A.P. J.Immunol.Methods 1987. 101: 109.
10. Shah, V.O., McCarley, D.L. and Weiner, R.S. Cryobiology. 1984. 21: 475.
11. Tax, W.J.M., Leeuwenberg, H.F.M., Willems, H.W., Capel, P.J.A. and Koene, R.A.P. in Bernard, A. et al. (Eds.), Human leucocyte differentiation antigens detected by monoclonal antibodies, Springer-Verlag, Berlin 1984, p. 721.
12. Beverley, P.C.L. and Callard, R.E. Eur.J.Immunol. 1981. 11: 329.
13. Dougherty, G.J., Selvendran, Y., Murdoch, S., Palmer, D.G. and Hogg, N. Eur.J.Immunol. 1987. 17: 1453.
14. Looney, R.J., Abraham, G.N. and Anderson, C.L. J.Immunol. 1986. 136: 1641.
15. Tetteroo, P.A.T., Van der Schoot, C.E., Visser, F.J., Bos, M.J.E. and Von dem Borne, A.E.G.Kr. in McMichael, A.J. et al. (Eds.), Leucocyte typing III, Oxford University Press, Oxford 1987, p. 702.
16. Phillips, J.H. and Babcock, G.F. Immunol.Letters 1983. 6: 143.
17. Thompson, J.G., Goeken, N.E., Brown, S.A. and Rhodes, J.L. in American association for clinical histocompatibility testing, 8th Annual Meeting 1982, p. A23.
18. Breard, J., Reinherz, E.L., Kung, P.C., Goldstein, G. and Schlossman, S.F. J.Immunol. 1980. 124: 1943.
19. Todd, III, R.F., Van Agthoven, A., Schlossman, S.F. and Terhorst, C. Hybridoma 1982. 1: 329.
20. Van de Winkel, J.G.J., Tax, W.J.M., Groeneveld, A., Tamboer, W.P.M., De Mulder, P.H.M. and Capel, P.J.A. J.Immunol.Methods 1988. 108: 95.
21. Perussia, B., Acuto, O., Terhorst, C., Faust, J., Lazarus, R., Fanning, V. and Trinchieri, G. J.Immunol. 1983. 130: 2142.
22. Frey, J. and Engelhardt, W. Eur.J.Immunol. 1987. 17: 583.

23. Cosio, F.G., Ackerman, S.K., Douglas, S.D., Friend, P.S. and Michael, A.F. Immunology 1981. 44: 773.
24. Hirschhorn, R., in Zweifach, B. (Ed.), The inflammatory process, Academic Press, New York 1974, p. 259.

CHAPTER 8

CONVERSION OF THE TYPE II Fc RECEPTOR FOR HUMAN IgG ON MONOCYTES FROM LOW TO HIGH AFFINITY BY PROTEOLYSIS

Jan G.J. van de Winkel, René van Ommen, Peter J.A. Capel, Robert A.P. Koene, and Wil J.M. Tax

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SUMMARY

The effect of proteolytic enzymes on the low-affinity Fc receptor for human IgG, FcγRII, present on human monocytes, was investigated. For this purpose, the high-affinity Fc receptor, FcγRI was selectively modulated from the monocytes using immobilized immune complexes. Proteolysis of such modulated monocytes induced an increased interaction with erythrocytes sensitized with human IgG (EA-human IgG). FcγRII appears to mediate this interaction. This conclusion is supported by the observation that after proteolysis, the FcγRII-mediated binding of EA-mouse IgG1 becomes susceptible to inhibition by (monomeric) human IgG. Studies on the effect of proteolytic enzymes were also performed with cell line K562, which expresses only FcγRII and not FcγRI. A dramatic increase in rosetting with EA-human IgG was observed when K562 cells were treated with proteases, and this increase could be inhibited by anti-FcγRII monoclonal antibodies. The expression of FcγRII was not influenced by proteolysis as evidenced by immunofluorescence studies, and neither were FcγRI or FcγRIII induced. We conclude that proteolysis increases the affinity of FcγRII for human IgG, and we speculate that such a proteolysis-induced increase of affinity might also occur in vivo, e.g. at inflammatory sites.

1. INTRODUCTION

Human monocytes constitutively possess two types of receptors for the Fc portion of IgG, FcγRI and FcγRII. FcγRI has an approximate molecular weight of 70 kDa and is defined by monoclonal antibodies (mAb) 32.2 (1) and 10.1 (2), whereas FcγRII has an approximate molecular weight of 40 kDa and is recognized - inter alia - by mAb IV.3 (3) and CIKM5 (4) (reviewed in 5). FcγRI binds both monomeric and aggregated or complexed human IgG with high affinity (5,6). FcγRII was defined originally by its interaction with mouse IgG1 (7,8). This receptor has low affinity for aggregated human IgG and no detectable affinity for monomeric human IgG (5,6). These receptors can be assayed in rosetting experiments by using erythrocytes sensitized either with human IgG (EA-human IgG; FcγRI) or with mouse IgG1 (EA-mouse IgG1; FcγRII) (3,9).

It is well known that FcγRI on human monocytes is trypsin (10) and pronase (11) resistant. The effects of proteolytic enzymes on FcγRII on human monocytes are unknown, and were investigated in this study. For this purpose, FcγRI was first modulated, by interaction with immobilized immune complexes, from the apical surface of the monocytes (12). Treatment of these modulated cells (which still bear FcγRII) with the proteolytic enzymes trypsin or pronase, increased the binding of EA-human IgG. This suggested that proteolysis increased the interaction of FcγRII with human IgG. Because of the complexity of experiments with monocytes, due to the presence of two types of FcγR, experiments were performed with K562 cells. These cells express only FcγRII and no FcγRI (3,5,6). Also on this cell line protease treatment induced increased interaction with human IgG, which could furthermore be inhibited by anti-FcγRII antibodies. Our results suggest that proteases can increase the affinity of FcγRII for human IgG. Since proteolytic enzymes can also be released in vivo, e.g. at inflammatory sites (13), we speculate that FcγRII might function in vivo as a "standby" Fc receptor for human IgG.

2. MATERIALS AND METHODS

2.1 Cells

Cytapheresis of healthy volunteers was applied to obtain white blood cell suspensions. Monocytes were isolated from these suspensions by Percoll (Pharmacia, Uppsala, Sweden) centrifugation and counterflow centrifugation as described before (14). Monocyte suspensions consisted for at least 90% of monocytes (evaluated in cytocentrifuge preparations after staining for non-specific esterase and with May-Grünwald-Giemsa), and viability was high (> 98%, assessed by trypan blue exclusion). Isolated cells were cryopreserved (15) and stored in liquid nitrogen until use. In some experiments we used the erythromyeloid cell line K562, which was cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, and gentamycin (50 µg/ml).

2.2 Anti-FcγR antibodies

Anti-FcγRI monoclonal antibodies used were 32.2 provided by Dr. C. Anderson (1), and 10.1 from Dr. N. Hogg (2). Two mAb with reactivity to FcγRII were used, IV.3 a gift from Dr. C. Anderson (3), and CIKM5 provided by Dr. G. Pilkington (4). The following anti-FcγRIII mAb were tested: anti-leu 11a (16), and anti-leu 11b (17), both from Becton Dickinson (Mountain View,Ca.).

2.3 FcγR-modulation

The method used for selective FcγRI-modulation was performed exactly as described previously (12). Briefly, flat-bottomed microtitre plates (Costar, Cambridge, Mass.) were coated with DNP-BSA (Calbiochem-Behring, La Jolla, Ca.), and subsequently incubated with PBS alone (Control), or with a saturating concentration of a chromatographically purified myeloma protein, reactive with DNP. Mouse IgG2a myeloma 5T2 (18) was provided by Dr. J. Radl. Monocytes were incubated in microtitre wells for 90 min at 37°C. After washing, cells were treated with proteolytic enzymes or with medium alone, and EA-rosetting was quantitated as described below.

2.4 Treatment with proteolytic enzymes

Monocyte-monolayers were incubated with RPMI 1640 medium alone (control) or with medium containing either 0.5 mg/ml trypsin, or 0.2 mg/ml pronase (both from Boehringer Mannheim, FRG) for 30 min at 37°C. After two washes, sensitized erythrocytes were added and binding was quantitated as described below. Viability of monocytes was high (> 95%, assessed by trypan blue exclusion) and was not decreased by protease treatment, nor was the number of cells per well influenced as evaluated by microscopal examination.

K562 cells were incubated in suspension (at a concentration of 4 x 10^6 cells/ml) with RPMI 1640 medium alone (control) or with medium containing either 0.5 mg/ml pronase, or 1 mg/ml trypsin for 90 min at 4°C. After three washes, cells were resuspended in RPMI 1640 medium containing 5% FCS and rosette formation was assayed as des-

cribed below. No substantial loss of cells was observed after treatment with proteases (5.2±4.4%, relative to cells incubated in medium alone; mean ± SD, nine individual experiments), and viability always exceeded 95%.

In some experiments protease inhibitors were used, which were preincubated for 30 min at room temperature with proteases, before addition to the cells. The following inhibitors were employed, soybean trypsin inhibitor (used in an amount of 0.5 mg per mg of trypsin), Nα-tosyl lysyl chloromethyl ketone (TLCK; used in a concentration of 0.8 mM), and phenylmethylsulphonyl fluoride (PMSF; used in a concentration of 0.4 mM). All inhibitors were obtained from Sigma (St.Louis, Mo.).

2.5 EA-rosetting

Two types of indicator cells were used for EA-rosette assays. Rhesus D-positive human erythrocytes were sensitized either with a human alloserum against rhesus D (Merz & Dade, Düdingen, Switzerland), or with a mouse IgG1 mAb against glycophorin A (obtained from M.Bos, CLB, Amsterdam). Sensitization was performed as described before (14). In some experiments the exact number of antibody molecules per HRBC was quantitated using a recently developed radiometric assay (19).

Binding of sensitized HRBC (and non-sensitized HRBC as a control for non-specific binding) to monocyte monolayers was evaluated after incubation for 1 h at 4°C. Quantitation of binding was performed photometrically at 450 nm using the pseudoperoxidase activity of HRBC as indicator (14). Binding indices were calculated, in order to correct for variations in background absorbance values between different experiments, as follows:
[1 - (E450/EA450)] x 100 = binding index, where E450 is extinction at 450 nm in wells to which HRBC without sensitizing antibody were added, and EA450 is extinction at 450 nm in wells to which sensitized HRBC were added. All determinations were performed in duplicate, and the variability was less than 10%.

In some experiments, enzyme-treated monocytes were incubated with monomeric human IgG for 15 min at 4°C, prior to addition of sensitized HRBC. Human IgG was purified from human serum by $(NH4)_2SO_4$ precipitation and DEAE-Sephadex (Pharmacia, Uppsala, Sweden) chromatography, consisted for more than 99.9% of human IgG as evaluated by immunoelectrophoresis and radial immunodiffusion, and was depleted of aggregates by ultracentrifugation (1 h at 100,000 g, 4°C). After incubation of monolayers with sensitized HRBC for 1 h at 4°C, binding was assayed photometrically, and percentages inhibition were calculated by comparing absorbances in the absence and presence of inhibitor, as described before (9).

Rosetting with K562 cells was scored microscopically, essentially as described before (14). Briefly, to 200 µl cells (at $2x10^6$/ml in

RPMI 1640 with 5% FCS), 50 µl sensitized HRBC suspension (0.5% v/v) were added, the samples were centrifuged 2 min at 20 g, and incubated for 1 h at 4°C. K562 cells with at least three bound HRBC were scored as rosettes, using a microscope with 400x final magnification. Unsensitized HRBC were included in each experiment, and were never found to form rosettes with K562 cells (neither protease-treated, nor untreated). At least 200 cells were scored per incubation. In inhibition experiments anti-FcγR mAb were added to K562 cells after incubation with proteases, experiments were further performed under similar conditions as above.

2.6 Immunofluorescence

The expression of FcγR on enzyme-treated cells was assayed by indirect immunofluorescence, using different anti-FcγR mAb and fluorescein-conjugated $F(ab')_2$ fraction of sheep anti-mouse IgG antibody (Cappel, Malvern, Pa.). Fluorescence was determined with an Ortho 50-H flow cytometer. In each sample the fluorescence intensity from 10,000 cells was measured.

3. RESULTS AND DISCUSSION

When monocytes are incubated in the presence of substrate-immobilized immune complexes containing mouse IgG2a antibody the subsequent binding of EA-human IgG is strongly decreased, while binding of EA-mouse IgG1 is hardly affected under these conditions (12). This suggests that the high-affinity FcγRI is modulated from the apical surface of the cells to the substrate-adherent side while FcγRII remains unaffected. When after such modulation monocytes were incubated with the protease trypsin, an increase in binding of EA-human IgG was observed (Fig.1). Monocyte binding of EA-mouse IgG1 was also found to be enhanced by trypsin. Similar results were obtained with the proteolytic enzyme pronase (data not shown). Since the high-affinity FcγRI was not detectable on the apical surface of monocytes after incubation with immune complex coated substrates, these results suggest that FcγRII can mediate interaction with EA-human IgG after treatment of monocytes with proteases. This conclusion was supported by the observation that monomeric human IgG nearly completely inhibited the rosetting between trypsin- or pronase-treated monocytes and EA-mouse IgG1, whereas untreated monocytes were not susceptible to such inhibition (Fig.2). Inhibition

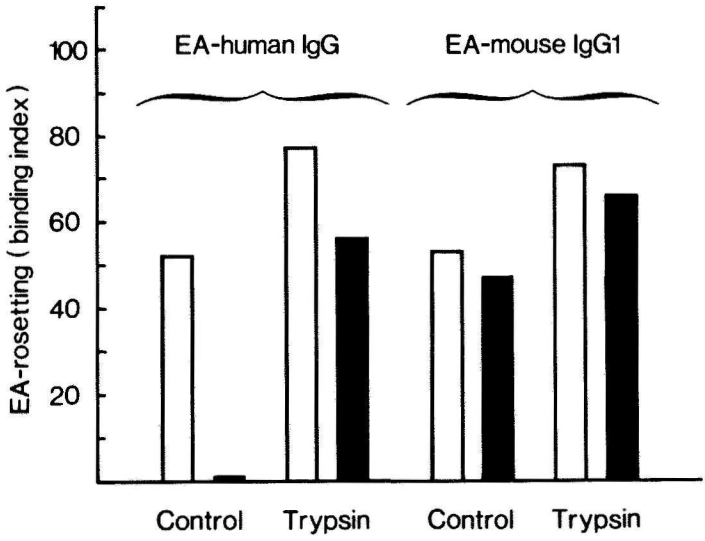

Fig.1. Effect of trypsin on monocyte-mediated binding of erythrocytes sensitized with human IgG or mouse IgG1, after incubation of monocytes with (■) or without (□) substrate-immobilized immune complexes. Monocyte-monolayers were incubated either with RPMI 1640 medium alone (control) or with medium containing trypsin, and after washing, binding of sensitized erythrocytes was assayed photometrically. Results are representative for seven experiments.

of (FcγRI-mediated) rosetting between monocytes and EA-human IgG by monomeric human IgG was not affected by treatment of monocytes with proteolytic enzymes.

Because the presence of two types of FcγR on monocytes makes studies with these cells relatively complex, we extended these observations using a cell line, K562 which expresses only FcγRII (3,5,6). This human cell line has a very low affinity for human IgG (6) and hardly forms EA-rosettes with human IgG-sensitized erythrocytes (9,14). EA-rosetting between K562 cells and erythrocytes sensitized with human IgG could be increased markedly by treatment of K562 with proteases (Fig.3a). The number of human IgG molecules per

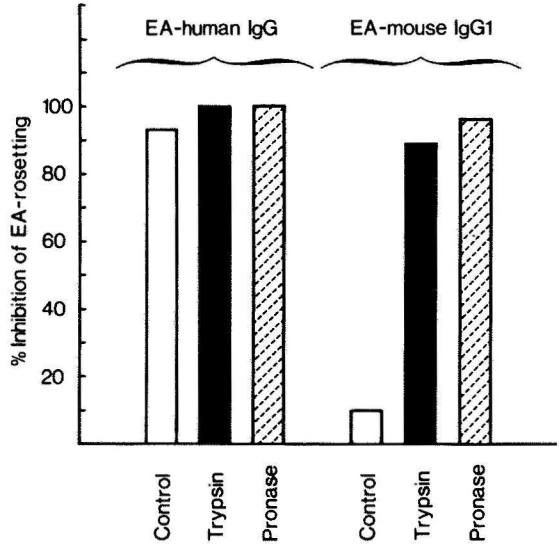

Fig.2. Inhibition by monomeric human IgG of rosette formation between sensitized erythrocytes and monocytes. Monocyte-monolayers in microtitre wells were incubated either with RPMI 1640 medium only (control) or with medium containing either trypsin, or pronase. After washing, binding of sensitized erythrocytes was assayed photometrically. This experiment was repeated five times with comparable results.

erythrocyte necessary to obtain half-maximal rosetting was observed to be approximately 5,000. This is similar to the values reported for EA-rosetting between (untreated) monocytes and human IgG-sensitized erythrocytes (19,20). The rosette formation between enzyme-treated K562 cells and EA-human IgG could be inhibited completely by different monoclonal anti-FcγRII antibodies, but not by monoclonal antibodies directed against FcγRI (Fig.3b). Monoclonal antibodies IV.3 and CIKM5 have previously been shown to block only EA-rosetting between human leukocytes and EA-mouse IgG1, and not rosette formation with EA-human IgG (3,4,9). These antibodies recognize different epitopes on FcγRII (Dr. Tetteroo, personal communication). It has been demonstrated previously that monoclonal anti-FcγRI antibody 10.1 completely inhibits the rosette formation

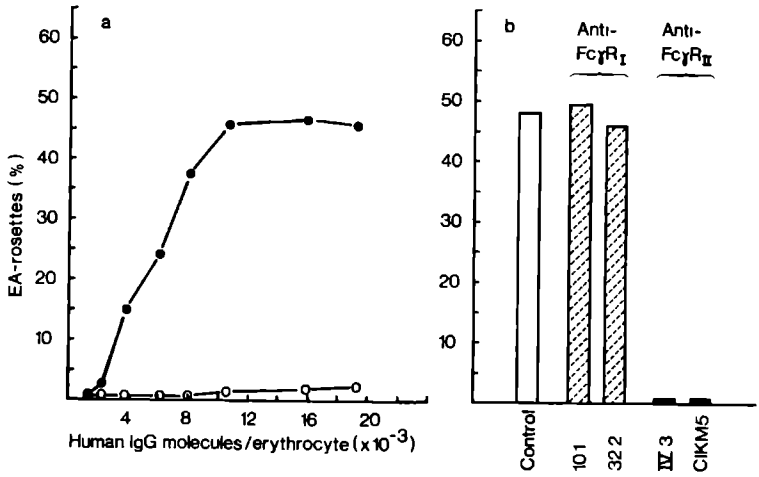

Fig.3. Rosette formation between human IgG-sensitized erythrocytes and K562 cells. a) K562 cells were incubated in the absence (o) or presence (•) of pronase, washed and rosetting with erythrocytes, sensitized with a range of human IgG molecules/cell was scored by a microscope; b) after pronase incubation, either RPMI 1640 medium alone (control) or medium supplemented with an anti-Fcγ receptor antibody was added, and rosetting was evaluated with optimally sensitized indicator erythrocytes i.e. with 10,600 human IgG molecules/erythrocyte. Experiments were repeated at least thrice using both pronase and trypsin (data not shown) with comparable results.

against an epitope on FcγRI distinct from the ligand binding site (1), and was used as a control antibody.

The effect of proteolytic enzymes on the expression of Fc receptors by K562 was studied using immunofluorescence analysis. Proteolysis had no effect on the expression of FcγRII, as evaluated by using two anti-FcγRII monoclonal antibodies (Fig.4). Expression of FcγRI could not be observed on protease-treated K562 cells using monoclo-

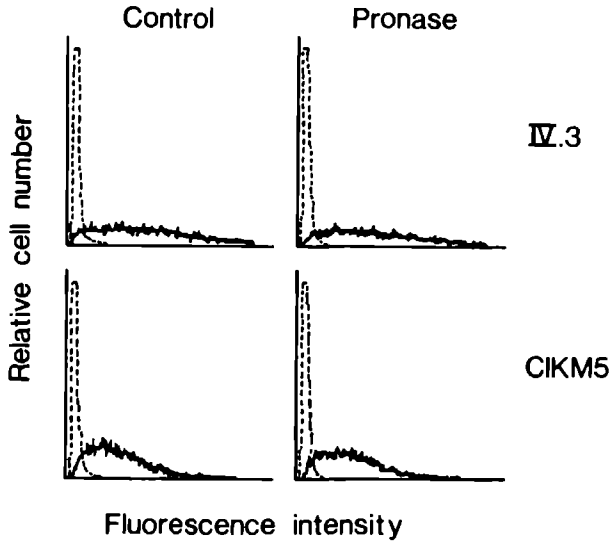

Fig.4. Immunofluorescence analysis of FcγRII on K562 cells after pronase treatment. K562 cells were incubated in RPMI 1640 medium alone (control) or medium containing pronase. After washing, the expression of the low-affinity FcγR was evaluated with a flowcytometer. Fluorescence was recorded as arbitrary units on a linear scale, using RPMI 1640 medium containing 10% FCS alone as a control for non-specific binding (dashed line). This experiment was repeated twice, employing both pronase and trypsin (data not shown) with comparable results.

could not be observed on protease-treated K562 cells using monoclonal antibodies 10.1 and 32.2 directed against two different antigenic determinants on this receptor (1,2), neither was a third type of FcγR (FcγRIII) expressed on K562 cells (with or without incubation in the presence of proteolytic enzymes). This third type of FcγR is expressed by human macrophages, granulocytes, and certain types of lymphocytes, and is defined by several antibodies belong-

ing to the CD16 cluster (4,5). The anti-CD16 antibodies tested by us were Anti-Leu11a (16) and Anti-Leu11b (17). On monocytes, no increase in FcγRI or II expression was found after enzyme-treatment, and no induction of FcγRIII was detectable (data not shown). Since the expression of FcγRII does not increase by protease treatment and the binding of both monomeric and complexed human IgG increases, the most likely explanation for these observations is that the affinity of FcγRII for human IgG increases considerably. This hypothesis was supported by the finding that binding of both monomeric as well as heat-aggregated human IgG was observed to increase sharply after protease treatment of K562 cells (data not shown).

Experiments with different enzyme inhibitors showed that the enzymatic activity of both trypsin (demonstrated by using soybean trypsin inhibitor and TLCK) and pronase (by employing PMSF) is essential for the observed effects on FcγR (data not shown).

In conclusion, we found that proteolytic enzymes can influence the degree of interaction of human IgG with FcγR. The most dramatic effects were observed for the low-affinity FcγRII on human monocytes of which the interaction with human IgG (both monomeric and complexed) increased profoundly after interaction with proteases. As the expression of FcγRII was found to remain stable it is likely that the affinity increases drastically. It has previously been reported (11,21), that trypsin and pronase treatment induces an increase in the affinity of human monocytes for rabbit IgG immune complexes, without affecting the number of binding sites for rabbit IgG. At the time, FcγRII had not yet been discovered. It seems likely that these effects are also based on the increased affinity of FcγRII as we have observed in the current study. Previously described effects of modulatory conditions (for instance the effect of Interferon-γ on monocyte FcγRI) primarily influenced the number of receptors and not their affinity for ligand (6). We speculate that in vivo proteolytic enzymes can have an important effect on the function of human monocyte FcγRII, because high concentrations of

proteolytic enzymes have been reported for instance in areas of inflammation (13). It has furthermore been shown that interaction of immune complexes with macrophages can induce the release of proteases (22). Another source of proteolytic enzymes could be activated T cells (23). Recently it was reported that Con A supernatants from human mononuclear cells induced an increased EA-rabbit IgG rosetting of human monocytes, which could be inhibited by serine esterase inhibitors (e.g. PMSF) (24).

4. CONCLUDING REMARKS

We think that our results point to the importance of the low-affinity FcγRII on human monocytes for the immune system. The physiological significance of FcγRI has been questioned before (2), since this receptor has a high affinity for monomeric human IgG (Ka= 10^8-10^9 M^{-1}) (4,5) and is likely to be saturated with human IgG which is present at approximately 10^{-5} M in serum. Furthermore, Ceuppens et al. (25) have described a family with deficiency of FcγRI without any evidence of disease symptoms or serological abnormalities. FcγRII on the other hand will not be blocked by human serum IgG. This structure might function as a "standby" Fc receptor for IgG with a negligible affinity for human IgG under normal conditions and an increase of this affinity by the activity of proteolytic enzymes, released locally during an inflammatory response.

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5. REFERENCES

1. Anderson, C.L., Guyre, P.M., Whitin, J.C., Ryan, D.H., Looney, R.J. and Fanger, M.W. J.Biol. Chem. 1986. 261: 12856.
2. Dougherty, G.J., Selvendran, Y., Murdoch, S., Palmer, D.G. and Hogg, N. Eur.J.Immunol. 1987. 17: 1453.
3. Looney, R.J., Abraham, G.N. and Anderson, C.L. J.Immunol. 1986. 136: 1641.
4. Tetteroo, P.A.T., Van der Schoot, C.E., Visser, F.J., Bos, M.J.E. and Von dem Borne, A.E.G.Kr. in Mc Michael, A.J. et al. (Eds.), Leucocyte Typing III, Oxford University Press, Oxford 1987, p. 702.
5. Anderson, C.L. and Looney, R.J. Immunol.Today. 1986. 7: 264.
6. Perussia, B., Dayton, E.T., Lazarus, R., Fanning, V. and Trinchieri, G. J.Exp.Med. 1983. 158: 1092.
7. Tax, W.J.M., Willems, H.W., Reekers, P.P.M., Capel, P.J.A. and Koene, R.A.P. Nature 1983. 304: 445.
8. Tax, W.J.M., Hermes, F.F.M., Willems, H.W., Capel, P.J.A. and Koene, R.A.P. J.Immunol. 1984. 133: 1185.
9. Van de Winkel, J.G.J., Tax, W.J.M., van Bruggen, M.C.J., van Roozendaal, C.E.P., Willems, H.W., Vlug, A., Capel, P.J.A. and Koene, R.A.P. Scand.J.Immunol. 1987. 26: 663.
10. LoBuglio, A.F., Cotran, R.S. and Jandl, J.H. Science 1967. 158: 1582.
11. Cosio, F.G., Douglas, S.D. and Michael, A.F. Immunology 1982. 46: 395.
12. Van de Winkel, J.G.J., van Duijnhoven, J.L.P., van Ommen, R., Capel, P.J.A. and Tax, W.J.M. J.Immunol. 1988. 140: 3515.
13. Hirschhorn, R., in Zweifach, B. (Ed.), The inflammatory process, Academic press, New York 1974, p. 259.
14. Van de Winkel, J.G.J., Tax, W.J.M., Van Bruggen, M.C.J., Van Roozendaal, C.E.P., Willems, H.W., Van Duijnhoven, J.L.P., Capel, P.J.A. and Koene, R.A.P. J.Immunol.Methods. 1987. 101: 109.
15. Shah, V.O., McCarley, D.L. and Weiner, R.S. Cryobiology 1984. 21: 475.
16. Phillips, J.H. and Babcock, G.F. Immunol.Letters 1983. 6: 143.
17. Thompson, J.G., Goeken, N.E., Brown, S.A. and Rhodes, J.L. in American Association for Clinical Histocompatibility Testing, 8th Annual Meeting 1982, p. A23.
18. Radl, J., Croese, J.W., Zurcher, C., Van den Enden-Vieveen, M.H.M., Brandijk, R.J., Kazil, M., Haayman, J.J., Reitsma, P.H. and Bijvoet, O.L.M. Cancer 1985. 55: 1030.
19. Van de Winkel, J.G.J., Tax, W.J.M., Groeneveld, A., Tamboer, W.P.M., De Mulder, P.H.M. and Capel, P.J.A. J.Immunol.Methods 1988. 108: 95.
20. Kurlander, R.J., Rosse, W.F. and Logue, G.L. J.Clin.Invest. 1978. 61: 1309.
21. Cosio, F.G., Ackerman, S.K., Douglas, S.D., Friend, P.S. and Michael, A.F. Immunology 1981. 44: 773.

23. Fruth, U., Sinigaglia, F., Schlesier, M., Kilgus, J., Kramer, M.D. and Simon, M.M. Eur.J.Immunol. 1987. 17: 1625.
24. Sipka, S., Kávai, M., Teichmann, F., Boldogh, I. and Szegedi, Gy. Haematologia 1987. 20: 73.
25. Ceuppens, J.L., Meurs, L. and Van Wauwe, J.P. J.Immunol. 1985. 134: 1498.

CHAPTER 9

DISCUSSION AND SUMMARY

In this thesis we have studied in detail human monocyte receptors for the Fc moiety of IgG, FcγR receptors, with special emphasis on the heterologous interactions between human FcγR and mouse immunoglobulins. This latter aspect seems important in view of the growing interest in the application of mouse monoclonal antibodies in immunotherapy. A brief overview of the distribution and functions of FcR is given in Chapter 1.

In Chapter 2 we describe a new method for the characterization of Fc receptors on human monocytes and cell lines by EA-rosetting. Binding of sensitized erythrocytes to monolayers of cells in microtitre plates is quantitated by employing the pseudoperoxidase activity of erythrocytes in a photometric way. We furthermore studied the amount of negative charge on different types of indicator erythrocytes, by an alcian blue binding assay (1), which appears to be an important parameter in this type of EA-rosette assays. Human erythrocytes were observed to possess a much higher membrane negative charge than either sheep or rabbit erythrocytes, which may be advantageous in EA-rosette methods by lowering the degree of non-specific binding (and thus increasing the signal to noise ratio).

The different types of FcγR on human monocytes have been characterized by using the photometric assay (Chapter 3). Two types of FcγR could be distinguished by EA-rosetting. The first type of FcγR forms rosettes with human erythrocytes sensitized by human IgG (EA-human IgG, FcγRI). This type of rosetting could be inhibited by monomeric mouse (m)IgG2a and by all the human IgG subclasses. Another type of FcγR could be assayed by using erythrocytes sensitized with a mouse IgG1 antibody (EA-mouse IgG1, FcγRII). This receptor was observed to be specific for mIgG1 and does not bind monomeric mIgG2a or mIgG2b nor, surprisingly, human IgG. These studies suggest the presence of two independent FcγR on human monocytes, which is further supported by the results obtained with human cell

lines, expressing either one or both types of FcγR. FcγRII, which interacts well with mIgG1, was studied in more detail, especially in view of the previously reported polymorphism in mitogenic effect of mouse IgG1 anti-CD3 mAb in humans. About 30% of Caucasian individuals (low-responders; LR) possess monocytes that do not support T cell proliferation by mIgG1 anti-CD3 antibodies, whereas monocytes from the remaining 70% (high-responder individuals; HR) support the mitogenesis very effectively (2). In previous studies using EA-mouse IgG1 rosetting it had been shown (3) that the polymorphism in mitogenic effect of mIgG1 anti-CD3 antibodies is due to a polymorphism of monocyte FcγRII. We now demonstrate that the difference between HR and LR individuals in binding of mouse IgG1 to FcγRII is quantitative rather than qualitative.

The affinities of the two types of FcγR were analyzed by comparing binding of indicator erythrocytes, sensitized with calibrated numbers of IgG molecules/cell, in EA-rosette assays (Chapter 4). Quantitation of the degree of erythrocyte sensitization was performed using a new type of radiometric assay. Erythrocytes which were sensitized with a human alloserum against rhesus D (EA-human IgG) bound maximally approximately 20,000 molecules/cell. The other type of indicator erythrocytes, which were constructed by using an anti-glycophorin A monoclonal antibody (EA-mouse IgG1), could contain up to 400,000 mIgG1 molecules per HRBC (without the occurrence of agglutination). When EA-rosette formation between monocytes and these indicator erythrocytes was evaluated, we found that half-maximal rosetting was obtained with EA-human IgG, sensitized with human IgG at less than 3,000 molecules/cell. At least 30 times more mouse IgG1 molecules/HRBC had to be present for half-maximal rosetting with HR monocytes. Since it was recently found that human monocytes express ± 36,000 FcγRII molecules, and ± 20,000 FcγRI molecules per cell (4), the affinity of FcγRII for its ligand (mouse IgG1) is much lower than the affinity of FcγRI for the ligand human IgG. LR monocytes were found to require indicator cells with approximately twice the number of mIgG1 molecules/HRBC for half-maximal

rosetting, compared to HR monocytes. This suggests that either far less FcγRII are present, or that the affinity of FcγRII on LR monocytes for mIgG1 is much lower than on HR monocytes. As anti-FcγRII mAb IV.3 has been observed to bind with similar intensity to both HR and LR monocytes (5), the second possibility seems most likely.

The interrelation between the two types of FcγR was analyzed by modulation studies described in Chapter 5. In these experiments we used substrate-immobilized immune complexes containing Ig of different classes and subclasses. Monocytes were incubated in microtitre wells containing immune complexes, and binding of the two types of indicator HRBC to the apical surface of the monocytes was then assayed by the photometric method. It has been shown previously that FcγR on mouse macrophages were modulated to the substrate-adherent part of the cells when these were incubated with immune complexes coated to a solid support (6). No effect on the binding of sensitized HRBC was found when monocytes were incubated with immune complexes containing mouse IgE, IgA or IgM, or $F(ab')_2$ fragments of rabbit IgG. High concentrations of immune complexes containing IgG of mouse, goat or rabbit were able to induce a decrease in binding of both types of sensitized erythrocytes, suggestive of co-modulation of FcγRI and FcγRII. At lower concentrations of immune complexes we observed more selective patterns of modulation. Under these conditions, immune complexes containing mIgG2a or rabbit IgG mainly affected the binding of EA-human IgG (FcγRI modulation), whereas mIgG1 or mIgG2b, as well as goat IgG preferently induced FcγRII-modulation. The removal of FcγRII from the apical surface of monocytes to the substrate adherent part was confirmed by using anti-FcγRII mAb IV.3. These studies indicate that both types of FcγR have affinity for all types of IgG tested, but that the degree of interaction differs. They furthermore show that the two types of FcγR are independently organized in the membrane, which makes selective regulation theoretically possible. Such regulation has already been shown by studies in which the immunomodulatory effects of interferon-γ were analyzed. This lymphokine selectively in-

creased the expression of FcγRI on monocytes, whereas FcγRII was not upregulated (7). The concept of two distinct types of FcγR on human monocytes has been established now, and their main characteristics are summarized in Table 1.

In Chapter 6, we studied the functional activity of both types of FcγR in antibody-dependent cell-mediated cytotoxicity (ADCC), using sensitized erythrocytes as target cells. We observed that EA-human IgG, as well as EA-mouse IgG1 were effectively killed by human monocytes, suggesting that both FcγR were cytotoxic trigger molecules. This was supported by inhibition studies using anti-FcγR mAb and different monomeric Ig preparations. Cytolysis of EA-human IgG was inhibited by both monomeric human IgG and mIgG2a, and also by anti-FcγRI mAb 10.1. In contrast, lysis of EA-mouse IgG1 was decreased by monomeric mIgG1 in a dose-dependent way and by two anti-FcγRII mAb, IV.3 and CIKM5 (reacting with different epitopes on FcγRII). The efficacy of cytotoxicity mediated by either of the FcγR was evaluated by using targets sensitized with calibrated numbers of IgG molecules. FcγRI was found to be much more effective than FcγRII in cytolysis of sensitized HRBC. Furthermore, the polymorphism of FcγRII described above was also reflected in the antibody-dependent cytotoxicity of monocytes.

In Chapter 7 we studied the effects of proteolysis os human monocyte FcγR.Neither trypsin,nor pronase caused a decrease in rosetting mediated by FcγRI.When the effects of protease-treatment on the polymorphic FcγRII were studied, we observed a dramatically increased interaction of LR monocytes with mouse IgG1.EA-rosetting between LR monocytes and EA-mouse IgG1 was enhanced considerably by proteolysis, up to the level exhibited by HR monocytes.As the number of FcγR was not increased (as measured by immunofluorescense using anti-FcγR mAb), the affinity for this ligand must be increased by proteolysis. An enhancing effect of protease-treatment could also be demonstrated with HR monocytes,when sub-optimally sensitized indicator HRBC were used, both in EA-rosetting as well as in ADCC.

Table 1. Characteristics of FcγR receptors expressed on human monocytes

Characteristics	FcγRI	Ref.	FcγRII	Ref.
Molecular weight	72 kDa	(8)	40 kDa	(5,9)
Distribution:		(8,10-13)		(5,9,14,15)
- peripheral blood	monocytes (neutrophils, after induction)		monocytes, B cells, thrombocytes, neutrophils, eosinophils	
- cell lines	HL-60,U937		HL-60, U937, K562 Daudi, Raji	
Number of sites/cell	$1\text{-}4\times10^4$	(4,16-18)	$10^3\text{-}4\times10^4$	(4,19)
Affinity for human IgG	$Ka=10^8\text{-}10^9 M^{-1}$	(16-18)	$Ka=10^5\text{-}10^6 M^{-1}$	(20)
FcγR-binding site on IgG	CH2 domain	(21,22)	ND[a]	
Specificity for human IgG subclasses	hIgG3>hIgG1>hIgG4 >>hIgG2	(16,17, 23,24)	hIgG1>hIgG2,hIgG4>> hIgG3	(20)
Specificity for mouse IgG subclasses	mIgG2a=mIgG3>> mIgG1,mIgG2b	(14,16, 25-27)	mIgG1>mIgG2b>> mIgG2a,mIgG3	(5,14,26,27)
Crosslinking of anti-CD3 antibodies:		(2,5,13)		(2,3,5,28)
- isotype	mIgG2a (OKT3,WT32)		mIgG1 (Leu-4,WT31, UCHT1)	
- % individuals supporting mitogenesis	± 100%[b]		± 70% of Caucasian individuals, ± 15% of Asian individuals	
Monoclonal anti-FcγR antibodies	32.2,10.1	(11,13)	IV.3,CIKM5,2E1,KuFc79	(5,29-31)

a. ND: not determined
b. world-wide only one "defective" family reported (32)

The studies on the effects of proteolysis on FcγRII were extended in Chapter 8. We first selectively modulated FcγRI from monocytes by using immobilized immune complexes. Proteolysis of such modulated monocytes induced an increased interaction with erythrocytes sensitized with human IgG. Surprisingly, this interaction with human IgG appeared to be mediated by FcγRII. This explanation was supported by the observation that after protease-treatment binding of EA-mouse IgG1 to monocytes became susceptible to inhibition by monomeric human IgG. Because of the complexity of experiments with monocytes, due to the presence of two types of FcγR, we also performed experiments with K562 cells. These cells express only FcγRII and no FcγRI (5,10,33). A dramatic increase in rosetting with EA-human IgG was found when K562 cells were treated with proteases. This increase could furthermore be inhibited by anti-FcγRII antibodies. The expression of FcγRII on these cells was not influenced by proteolysis as evidenced by immunofluorescence studies, neither were other types of FcγR induced.

On the basis of these studies we have developed a new hypothesis on the role of the low-affinity FcγRII on human monocytes in the immune system. Due to their low affinity FcγRII will not be blocked by human IgG under normal conditions, in contrast to FcγRI (which possess an extremely high affinity for monomeric human IgG). However, FcγRII can be induced to bind hIgG under "special" conditions, when proteolytic enzymes are present. Elevated concentrations of proteolytic enzymes have been reported to be present at inflammatory sites (34). Furthermore, the release of proteases has also been observed after interaction of macrophages with immune complexes (35). Thus at inflammatory sites, the freely available FcγRII will acquire affinity for human IgG and will consequently be able to subserve in immunologic effector functions at these sites.

Recently, a promising new imaging technique has been described by Rubin et al. by which sites of focal inflammation in both animals and man can be localized. In this technique, ^{111}Indium-labelled

non-specific human IgG was injected into patients suspected of inflammation, and at defined intervals, gamma imaging was carried out. Focal sites of inflammation could be correctly identified in most patients, with a high specificity. This approach seems useful in many clinical situations, for instance for patients with possible intra-abdominal abscesses following surgery or trauma (36,37). The mechanism underlying this phenomenon is unknown. From our own results we postulate that modulation of FcγRII-affinity for human IgG by proteolysis may explain the selective localization of human IgG at the inflammatory site.

As can be read above, knowledge about human FcγR has rapidly increased during the last few years. To obtain a more thorough understanding of their structures and biological functioning, however, analysis of the genes encoding these molecules is essential. Three types of FcγR on human cells have been cloned up to now, which revealed that they are all members of the immunoglobulin superfamily. FcγRII was the first human FcγR to be cloned. At the DNA level this receptor was homologous to the mouse macrophage FcγRII. This receptor, which has been given the cluster of differentiation assignment CDw32, was found to possess two extracellular immunoglobulin-like domains. Transfectants expressing the receptor bound human IgG with a very low affinity which confirms earlier studies (20, 38,39). Another type of human FcγR, FcγRIII (CD16) has also been cloned recently. This type of FcγR is expressed by a variety of cells, amongst which are neutrophils, large granular lymphocytes, and macrophages, but not monocytes (33). This receptor also possesses an extracellular part containing two immunoglobulin-related segments. It expresses a low affinity for IgG upon transfection to COS cells (Ka= $5x10^5M^{-1}$). Most remarkably, this work revealed an unusual type of membrane anchor for this receptor, which was linked via a phosphatidylinositol glycan (40). The high-affinity FcγRI was also reported to be cloned by Dr. David Simmons (Massachusetts General Hospital), and contains three immunoglobulin-like extracellular domains. Upon transfection, this molecule bound human IgG with

the expected high affinity thus confirming that its function was embodied in this single molecule (41). Also from another type of recombinant DNA technology intriguing new approaches to the unravelling of FcγR-properties are possible. These studies use site-directed mutagenesis to modify immunoglobulins at the genetic level, and by such experiments the exact binding site on IgG for FcγRI could be attributed to only one single amino acid in the CH_2 domain (22).

By these new types of experimental approaches our insight into the structure of the immune system will increase enormously which will hopefully also benefit human patients. Such studies will certainly also have consequences for our understanding of the function of these receptors. One example of this is our new hypothesis on the function of low affinity FcγRII at inflammatory sites. This theory must be validated by subsequent studies, but if it proves to be correct, it can serve as a model of how the mononuclear phagocyte system exerts its important defense function in the inflammatory process.

REFERENCES

1. Van de Winkel, J.G.J., Wetzels, J.F.M., Van Duijnhoven, J.L.P., Koene, R.A.P., and Capel, P.J.A. Red blood cell surface charge and alcian blue binding. Nephrol.Dial.Transplant. 1987; 2: 280-281.
2. Tax, W.J.M., Willems, H.W., Reekers, P.P.M., Capel, P.J.A., and Koene, R.A.P. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. Nature 1983; 304: 445-447.
3. Tax, W.J.M., Hermes, F.F.M., Willems, H.W., Capel, P.J.A., and Koene, R.A.P. Fc receptors for mouse IgG1 on human monocytes: polymorphism and role in antibody-induced T cell proliferation. J.Immunol. 1984; 133: 1185-1189.
4. Selvaraj, P., Rosse, W.F., Silber, R., and Springer, T.A. The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria. Nature 1988; 333: 565-567.
5. Looney, R.J., Abraham, G.N., and Anderson, C.L. Human monocytes and U937 cells bear two distinct Fc receptors for IgG. J.Immunol. 1986; 136: 1641-1647.
6. Michl, J., Pieczonka, M.M., Unkeless, J.C., and Silverstein, S.C. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. J.Exp.Med. 1979; 150: 607-621.
7. Graziano, R.F., and Fanger, M.W. FcγRI and FcγRII on monocytes and granulocytes are cytotoxic trigger molecules for tumor cells. J.Immunol. 1987; 139: 3536-3541.
8. Anderson, C.L. Isolation of the receptor for IgG from a human monocyte cell line (U937) and from human peripheral blood monocytes. J.Exp.Med. 1982; 156: 1794-1805.
9. Rosenfeld, S.I., Looney, R.J., Leddy, J.P., Phipps, D.C., Abraham, G.N., and Anderson, C.L. Human platelet Fc receptor for immunoglobulin G. Identification as a 40,000-molecular weight membrane protein shared by monocytes. J.Clin.Invest. 1985; 76: 2317-2322.
10. Perussia, B., Dayton, E.T., Lazarus, R., Fanning, V., and Trinchieri, G. Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells. J.Exp.Med. 1983; 158: 1092-1113.
11. Anderson, C.L., Guyre, P.M., Whitin, J.C., Ryan, D.H., Looney, R.J., and Fanger, M.W. Monoclonal antibodies to Fc receptors for IgG on human mononuclear phagocytes. Antibody characterization and induction of superoxide production in a monocyte cell line. J.Biol.Chem. 1986; 261: 12856-12864.
12. Shen, L., Guyre, P.M., and Fanger, M.W. Polymorphonuclear leukocyte function triggered through the high affinity Fc receptor for monomeric IgG. J.Immunol. 1987; 139: 534-538.
13. Dougherty, G.J., Selvendran, Y., Murdoch, S., Palmer, D.G., and Hogg, N. The human mononuclear phagocyte high-affinity Fc receptor, FcγRI, defined by a monoclonal antibody, 10.1. Eur.J. Immunol. 1987; 17: 1453-1459.

14. Jones, D.H., Looney, R.J., and Anderson, C.L. Two distinct classes of IgG Fc receptors on a human monocyte line (U937) defined by differences in binding of murine IgG subclasses at low ionic strength. J.Immunol. 1985; 135: 3348-3353.
15. Looney, R.J., Ryan, D.H., Takahashi, K., Fleit, H.B., Cohen, H.J., Abraham, G.N., and Anderson, C.L. Identification of a second class of IgG Fc receptors on human neutrophils. A 40 kilodalton molecule also found on eosinophils. J.Exp.Med. 1986; 163: 826-836.
16. Anderson, C.L., and Abraham, G.N. Characterization of the Fc receptor for IgG on a human macrophage cell line, U937. J.Immunol. 1980; 125: 2735-2741.
17. Kurlander, R.J., and Batker, J. The binding of human immunoglobulin G1 monomer and small, covalently cross-linked polymers of immunoglobulin G1 to human peripheral blood monocytes and polymorphonuclear leucocytes. J.Clin.Invest. 1982; 69: 1-8.
18. Fries, L.F., Mullins, W.W., Cho, K.R., Plotz, P.H., and Frank, M.M. Monocyte receptors for the Fc portion of IgG are increased in systemic lupus erythematosus. J.Immunol. 1984; 132: 695-700.
19. Karas, S.P., Rosse, W.F., and Kurlander, R.J. Characterization of the IgG-Fc receptor on human platelets. Blood 1982; 60: 1277-1281.
20. Stengelin, S., Stamenkovic, I., and Seed, B. Isolation of cDNAs for two distinct human Fc receptors by ligand affinity cloning. EMBO J. 1988; 7: 1053-1059.
21. Partridge, L.J., Woof, J.M., Jefferis, R., and Burton, D.R. The use of anti-IgG monoclonal antibodies in mapping the monocyte receptor site on IgG. Molec.Immun. 1986; 23: 1365-1372.
22. Duncan, A.R., Woof, J.M., Partridge, L.J., Burton, D.R., and Winter, G. Localization of the binding site for the human high-affinity Fc receptor on IgG. Nature 1988; 332: 563-564.
23. Woof, J.M., Partridge, L.J., Jefferis, R., and Burton, D.R. Localization of the monocyte-binding region on human immunoglobulin G. Molec.Immun. 1986; 23: 319-330.
24. Zupanska, B., Thomson, E.E., and Merry, A.H. Fc receptors for IgG1 and IgG3 on human mononuclear cells - an evaluation with known levels of erythrocyte-bound IgG. Vox.Sang. 1986; 50: 97-103.
25. Lubeck, M.D., Steplewski, Z., Baglia, F., Klein, M.H., Dorrington, K.J., and Koprowski, H. The interaction of murine IgG subclass proteins with human monocyte Fc receptors. J.Immunol. 1985; 135: 1299-1304.
26. Van de Winkel, J.G.J., Tax, W.J.M., Van Bruggen, M.C.J., Van Roozendaal, C.E.P., Willems, H.W., Vlug, A., Capel, P.J.A., and Koene, R.A.P. Characterization of two Fc receptors for mouse immunoglobulins on human monocytes and cell lines. Scand.J.Immunol. 1987; 26: 663-672.
27. Van de Winkel, J.G.J., Van Duijnhoven, J.L.P., Van Ommen, R., Capel, P.J.A., and Tax, W.J.M. Selective modulation of two human monocyte Fc receptors for IgG by immobilized immune complexes. J.Immunol. 1988; 140: 3515-3521.

28. Abo, T., Tilden, A.B., Balch, C.M., Kumagai, K., Troup, G.M., and Cooper, M.D. Ethnic differences in the lymphocyte proliferative response induced by a murine IgG1 antibody, Leu-4, to the T3 molecule. J.Exp.Med. 1984; 160: 303-309.
29. Vaughn, M., Taylor, M., ,and Mahanakumar, T. Characterization of human IgG Fc receptors. J.Immunol. 1985; 135: 4059-4065.
30. Tetteroo, P.A.T., Van der Schoot, C.E., Visser, F.J., Bos, M.J.E., and Von dem Borne, A.E.G.Kr. Three different types of Fcγ receptors on human leucocytes defined by workshop antibodies; $Fc\gamma R_{low}$ of neutrophils, $Fc\gamma R_{low}$ of K/NK lymphocytes, and FcγRII. In Leucocyte Typing III. McMichael, A.J. et al., eds. Oxford University Press, Oxford. 1987. P. 702-706.
31. Willis, H.E., Browder, B., Feister, A.J., Mohannakumar, T., and Ruddy, S. Monoclonal antibody to human IgG Fc receptors. Cross-linking of receptors induces lysosomal enzyme release and superoxide generation by neutrophils. J.Immunol. 1988; 140: 234-239.
32. Ceuppens, J.L., Meurs, L., and Van Wauwe, J.P. Failure of OKT3 monoclonal antibody to induce lymphocyte mitogenesis: a familial defect in monocyte helper function. J.Immunol. 1985; 134: 1498-1502.
33. Anderson, C.L., and Looney, R.J. Human leucocyte IgG Fc receptors. Immunol.Today 1986; 7: 264-266.
34. Hirschhorn, R. Lysosomal mechanisms in the inflammatory process. In the inflammatory process. Zweifach, B., ed. Academic Press, New York. 1974. P. 259-285.
35. Cardella, C.J., Davies, P., and Allison, A.L. Immune complexes induce selective release of lysosomal hydrolases from macrophages. Nature 1974; 247: 46-48.
36. Rubin, R.H., Young, L.S. Hansen, W.P., Nedelman, M., Wilkinson, R., Nelles, M.J., Callahan, R., Khaw, B-A., and Strauss, H.W. Specific and nonspecific imaging of localized Fisher immunotype 1 pseudomonas aeruginosa infection with radiolabelled monoclonal antibody. J.Nucl.Med. 1988; 29: 651-656.
37. Fischman, A.J., Keech, F., Khaw, B.A., Callahan, R.J., Wilkinson, R., Rubin, R.H., and Strauss, H.W. Clinical experience with indium-111 non-specific human polyclonal IgG. J.Nucl.Med. 1988; 29: 830.
38. Stuart, S.G., Trounstine, M.L., Vaux, D.J.T., Koch, T., Martens, C.L., Mellman, I., and Moore, K.W. Isolation and expression of cDNA clones encoding a human receptor for IgG (FcγRII). J.Exp.Med. 1987; 166: 1668-1684.
39. Hibbs, M.L., Bonadonna, L., Scott, B.M., McKenzie, I.F.C., and Hogarth, P.M. Molecular cloning of a human immunoglobulin G Fc receptor. Proc.Natl.Acad.Sci.USA 1988; 85: 2240-2244.
40. Simmons, D., and Seed, B. The Fcγ receptor of natural killer cells is a phospholipid-linked membrane protein. Nature 1988; 333: 568-570.
41. Hogg, N. The structure and function of Fc receptors. Immunol. Today 1988; 9: 185-187.

SAMENVATTING

In dit proefschrift is onderzoek beschreven aan een type receptoren dat een cruciale rol speelt in het immuunsysteem van de mens. Deze receptoren kunnen een binding aangaan met het Fc-deel van immuunglobulinen en na binding kan een veelvoud van biologische effecten optreden. De studies concentreren zich rond receptoren voor IgG, FcγR, op humane monocyten, waarbij met name de interacties met muize antistoffen zijn bestudeerd. Dit laatste aspect lijkt vooral van belang omdat heterocytophiele interacties een belangrijke rol zullen spelen wanneer monoclonale muize antistoffen worden gebruikt voor immuuntherapie bij de mens.

Een korte inleiding tot het onderwerp van dit proefschrift wordt gepresenteerd in hoofdstuk 1.

In hoofdstuk 2 wordt een nieuwe methode beschreven waarmee Fc receptoren op humane monocyten en cellijnen kunnen worden gekarakteriseerd met behulp van EA rozettering. Deze techniek maakt gebruik van de pseudoperoxidase activiteit van de indicator erythrocyten, die fotometrisch kan worden bepaald. Tevens worden experimenten beschreven waarmee de negatieve lading van de membraan van erythrocyten wordt bepaald in een bindingsassay die gebruik maakt van de kationische kleurstof Alcian Blauw. Erythrocyten van humane origine blijken een hoge negatieve lading te bezitten, die mogelijk bijdraagt aan hun geringe niet-specifieke binding, en daarmee aan hun uitstekende bruikbaarheid als indicatorcel in de fotometrische EA rozettering.

In hoofdstuk 3 worden de verschillende types FcγR op humane monocyten nader gekarakteriseerd met behulp van de fotometrische EA-rozettering. Een tweetal types FcγR kan worden onderscheiden, FcγRI die rozettering met humaan IgG-beladen erythrocyten (EA-humaan IgG) medieert, en FcγRII die erythrocyten beladen met muis IgG1 antistoffen (EA-muis IgG1) bindt. FcγRI blijkt affiniteit voor zowel

monomeer humaan IgG (van alle vier de subklassen), als voor muis IgG2a te bezitten, terwijl FcγRII alleen antistoffen van het muis IgG1 isotype goed lijkt te kunnen binden. FcγRII wordt nader geanalyseerd, omdat deze receptor betrokken is bij een polymorfisme in mitogeniciteit van muis IgG1 anti-CD3 antistoffen. Monocyten van het grootste deel (±70%) van de mensen in West-Europa blijken de inductie van T cel proliferatie door muis IgG1 anti-CD3 antistoffen te ondersteunen (zogenaamde "high-responder" individuen; HR), terwijl monocyten van de overige individuen dit niet doen ("low-responder" individuen; LR). Uit experimenten in dit hoofdstuk blijkt dat dit polymorfisme berust op een kwantitatief, en niet een absoluut verschil in binding van muis IgG1 aan FcγRII van HR en LR monocyten.

In hoofdstuk 4 wordt de affiniteit van beide FcγR voor hun liganden nader geanalyseerd in EA rozetteringsassays. Hierin wordt gebruik gemaakt van indicator erythrocyten beladen met nauwkeurig gekwantificeerde aantallen IgG moleculen. Bepaling van de mate van sensibilisatie gebeurt met een nieuwe radiometrische methode. Rozettering van monocyten met EA-humaan IgG blijkt reeds half-maximaal te zijn wanneer erythrocyten worden gebruikt met minder dan 3000 moleculen humaan IgG per erythrocyt. Voor half-maximale rozettering met EA-muis IgG1 zijn minstens 30 maal zoveel muis IgG1 moleculen per erythrocyt nodig. Dit wijst op aanzienlijke affiniteitsverschillen tussen beide types FcγR voor hun liganden. LR monocyten blijken verder ongeveer dubbel zoveel muis IgG1 moleculen per erythrocyt nodig te hebben als HR monocyten om dezelfde mate van rozettering te bereiken. Daar de expressie van FcγRII gelijk is op monocyten van HR en LR, wijst dit op een verschil in affiniteit van deze receptor tussen beide types monocyten.

In hoofdstuk 5 wordt de relatie tussen beide FcγR in detail geanalyseerd in modulatie experimenten. Daarin wordt gebruik gemaakt van substraat-geïmmobiliseerde immuuncomplexen. Wanneer de interactie van een FcγR met een geïmmobiliseerd immuuncomplex tot modulatie

van de receptor heeft gevoerd, zal de binding van antistof-beladen erythrocyten aan de bovenkant van de monocyten zijn gereduceerd. Uit deze studies is gebleken dat FcγR alleen affiniteit bezitten voor immuuncomplexen gemaakt met antistoffen van de IgG klasse en niet van andere klassen Ig. Hoge concentraties IgG-immuuncomplexen induceren co-modulatie van FcγRI en FcγRII, terwijl bij zeer lage concentraties selectieve modulatie wordt waargenomen. Dit wijst erop dat beide FcγR affiniteit bezitten voor elk van de IgG's, maar dat de mate van affiniteit voor elk van de IgG's verschilt. Tevens blijkt uit deze experimenten dat de receptoren onafhankelijk van elkaar zijn georganiseerd in de celmembraan van humane monocyten.

In hoofdstuk 6 wordt de functionele aktiviteit van beide types FcγR onderzocht in cytotoxiciteit van monocyten tegen gesensibiliseerde erythrocyten (antistof-afhankelijke celgemedieerde cytotoxie: ADCC). Zowel erythrocyten beladen met humaan IgG, als met muis IgG1, blijken te worden gelyseerd na incubatie met monocyten. Deze cytolyse blijkt door respectievelijk FcγRI en FcγRII te worden gemedieerd. Dit kon worden aangetoond door inhibitie-studies met monomere immuunglobulines van diverse isotypes en met anti-FcγR monoclonale antistoffen. De cytotoxische effectiviteit van beide types FcγR verschilt drastisch: FcγRI is veel effectiever dan FcγRII. Tevens komt de polymorfie van FcγRII ook tot uitdrukking in de cytotoxiciteit van monocyten tegen muis IgG1 gesensibiliseerde erythrocyten.

In hoofdstuk 7 worden de effecten van proteolytische enzymen op monocytaire FcγR onderzocht. Behandeling van monocyten met trypsine of pronase remt de (FcγRI-gemedieerde) rozetvorming met EA-humaan IgG niet. Wanneer de effecten van deze proteases op de polymorfe FcγRII worden geanalyseerd, blijkt er een sterke verhoging van rozettering van LR monocyten met EA-muis IgG1 plaats te vinden na proteolyse. Deze verhoging is dusdanig sterk dat de rozettering van met protease behandelde LR monocyten overeenkomt met die van onbe-

handelde HR monocyten. Omdat de expressie van FcγRII niet wordt verhoogd door proteolyse, (zoals blijkt uit immuunfluorescentie analyses) lijkt het aannemelijk dat de affiniteit van FcγRII voor muis IgG1 sterk toeneemt. Een potentiërend effect van proteolyse kan ook worden waargenomen met HR monocyten, wanneer sub-optimaal beladen erythrocyten worden gebruikt, zowel in EA-rozettering als in ADCC.

De effecten van proteolytische enzymen op FcγRII worden nader geanalyseerd in experimenten beschreven in hoofdstuk 8. In deze studie wordt FcγRI eerst selectief van monocyten gemoduleerd door incubatie van de cellen met substraat-geïmmobiliseerde immuuncomplexen. Proteolyse van deze monocyten blijkt een sterke verhoging in binding van EA-humaan IgG te induceren die opmerkelijk genoeg, door FcγRII wordt gemedieerd. Behandeling van monocyten met proteolytische enzymen leidt dus tot een sterke toename van de affiniteit van FcγRII voor humaan IgG. Deze hypothese wordt ondersteund door de bevinding dat na proteolytische behandeling de rozettering van monocyten met EA-muis IgG kan worden geremd door monomeer humaan IgG, een remming die niet optreedt bij onbehandelde cellen. Omdat experimenten met monocyten erg complex zijn, vanwege de gelijktijdige expressie van zowel FcγRI als FcγRII, werden ook studies met de cellijn K562 uitgevoerd. Deze cellijn exprimeert van beide receptoren alleen FcγRII. Ook hier wordt na proteolyse een dramatische toename van rozettering met humaan IgG-beladen erythrocyten gevonden, die bovendien volledig te remmen blijkt met monoclonale antistoffen gericht tegen FcγRII. Daar de expressie van FcγRII niet wordt verhoogd, noch enig ander type FcγR wordt geïnduceerd, lijkt ook op K562 de affiniteit van FcγRII voor humaan IgG sterk toe te nemen na behandeling met proteases. Deze bevindingen geven mogelijk een aanwijzing over de fysiologische betekenis van FcγRII. Aangezien proteolytische enzymen ook kunnen vrijkomen bij ontstekingsprocessen, is het denkbaar dat FcγRII fungeert als een receptor die onder normale condities geen affiniteit heeft voor monomeer humaan IgG (en dus ook niet verzadigd zal zijn door de hoge concentratie

IgG in serum), maar een hoge affiniteit voor humaan IgG verwerft tijdens en ter plaatse van een ontstekingsreactie.

Samenvattend blijkt uit dit onderzoek dat humane monocyten een tweetal Fcγ receptoren tot expressie brengen. Deze receptoren bezitten verschillende bindingskarakteristieken voor immuunglobulinen en zijn onafhankelijk van elkaar georganiseerd in de membraan. Beide structuren kunnen als "trigger-molecuul" in ADCC fungeren. FcγRII is een polymorfe structuur; het verschil in affiniteit voor muis IgG1 tussen HR en LR individuen blijkt van kwantitatieve, niet van kwalitatieve aard. Behandeling van LR monocyten met proteolytische enzymen induceert een verhoging van de affiniteit van FcγRII tot een niveau dat wordt aangetroffen bij (onbehandelde) monocyten van HR. In tegenstelling tot FcγRI, heeft FcγRII onder normale condities geen affiniteit voor monomeer humaan IgG. Deze affiniteit kan echter sterk worden verhoogd door behandeling van monocyten met proteolytische enzymen. Aangezien dergelijke enzymen ook in vivo in verhoogde concentraties worden aangetrofen, bijvoorbeeld in ontstekingsgebieden, zou FcγRII kunnen fungeren als een "standby" receptor voor humaan IgG.

DANKWOORD

Allen die hebben bijgedragen aan het tot stand komen van dit proefschrift wil ik van harte bedanken. Een aantal mensen verdienen een speciaal woord van dank, omdat zonder hun hulp en inzet dit proefschrift niet zijn huidige vorm had kunnen bereiken: alle medewerkers van de afdelingen Nierziekten en Bloedziekten en verder met name (alfabetisch): Ger Boonen, Mieke van Bruggen, Hans van Duijnhoven, Patricia Groenen, Arie Groeneveld, Cor Jacobs, Peter Janssen, Gaby Martens-Ticoalu, René van Ommen, Arie Pennings, André Peters, Aart Plas, Marianne Prenen, Kees van Roozendaal, Truus Rijke-Schilder, Wim Tamboer, Arjen Vlug en Riki Willems.

Tenslotte wil ik Ilse Hilgers-Biermans zeer hartelijk danken voor het snel en zorgvuldig typen van de artikelen en voor de attente wijze waarop het manuscript drukklaar werd gemaakt.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 1 maart 1961 te Venray. In 1979 werd het diploma gymnasium-β behaald aan scholengemeenschap Het Jerusalem in zijn geboorteplaats. Daarna studeerde hij Biologie aan de Katholieke Universiteit Nijmegen. In april 1985 werd het doctoraalexamen afgelegd (cum laude), met als hoofdvak Dierfysiologie en als bijvakken Immunologie en Biochemie.

Van mei 1985 tot mei 1988 was hij werkzaam als wetenschappelijk medewerker op de afdeling Nierziekten van het Sint Radboudziekenhuis (hoofd: Prof.dr. R.A.P. Koene), waar het hiervoor beschreven onderzoek werd verricht. Gedurende deze tijd leverde hij tevens een bijdrage aan het doctoraalonderwijs voor studenten in de scheikunde en in de biologie en het onderwijs aan studenten van het HLO.

In mei 1988 volgde de aanstelling als wetenschappelijk medewerker aan de Rijksuniversiteit Utrecht, op de afdeling Experimentele Immunologie (hoofd: Prof.dr. P.J.A. Capel).

STELLINGEN

I

Het verschil in specificiteit van de twee types Fcγ receptoren op humane monocyten voor de verschillende immuunglobuline G subklassen is niet absoluut, maar relatief.

II

De Fcγ receptoren op humane monocyten, FcγRI en FcγRII, kunnen beide antistof-afhankelijke cellulaire cytotoxie mediëren.

III

Aangezien blootstelling van monocyten aan proteolytische enzymen leidt tot een verhoogde interactie tussen FcγRII en humaan IgG, lijkt een functie als "standby" Fcγ receptor, die in ontstekingsgebieden kan worden geactiveerd, aannemelijk.

IV

Fcγ receptoren met een lage affiniteit voor monomeer humaan IgG zijn waarschijnlijk belangrijker voor de klaring van immuuncomplexen dan Fcγ receptoren met een hoge affiniteit.

V

Men dient rekening te houden met de mogelijkheid dat associatie van Fcγ receptoren met andere moleculen in de celmembraan essentieel is voor hun werking.

VI

De veronderstelling van Anderson en Looney dat FcγRIII door humane eosinophiele granulocyten wordt geëxprimeerd is onjuist.

Anderson CL, Looney RJ. Human leukocyte IgG Fc receptors. Immunol Today 1986; 7: 264-266

VII

Bij het definiëren van de specificiteit van anti-monocytaire antistoffen dient men zorgvuldig de reactiviteit met bloedplaatjes te controleren.

VIII

De bewering van Benichou et al., dat FcγRII ontbreekt op monocyten van "low-responder" individuen, is onjuist.

Benichou G, Kanellopoulos JM, Wallon C, Boue F, Delfraissy J-F. Interferon-γ restores T lymphocyte proliferation of nonresponders to IgG1 anti-CD3 via the induction of $Fc\gamma_1$ receptors on monocytes. Eur J Immunol 1987; 17: 1175-1181

IX

Het lijkt aannemelijk dat glycosaminoglycanen in het slijm van vissen een functie hebben als "calcium reservoir", daar de concentratie van deze stoffen toeneemt na adaptatie aan water met een laag calciumgehalte.

Van de Winkel JGJ, Van Kuppevelt AHMSM, Janssen HMJ, Lock RAC. Glycosaminoglycans in the skin mucus of rainbow trout (Salmo Gairdneri). Comp Biochem Physiol 1986; 85B: 473-475

X

Experimenten waarin cellen langdurig worden geïncubeerd met calcium ionofoor zijn niet betrouwbaar, vanwege het cytotoxische karakter van de testcondities.

Yu C-L, Tsai M-H, Stacey DW. Cellular ras activity and phospholipid metabolism. Cell 1988; 52: 63-71

XI

De toxicologische bezwaren tegen het gebruik van conserveermiddelen in voedingsmiddelen worden in de publieke meningsvorming onvoldoende afgewogen tegen de risico's van het niet toepassen van deze middelen.

XII

Het huidige vestigingsbeleid van tandartsen en huisartsen dient drastisch te worden gesaneerd.

XIII

Standaardisering van de tekst op de bij verkeersborden gebruikte onderborden zal de verkeersveiligheid ten goede komen.

XIV

UV-A bruiningsapparaten werken zelf verkleining van het afzetgebied voor dit soort apparatuur in de hand.

Nijmegen, 7 october 1988 Jan G.J. van de Winkel