

# A NEW Fc RECEPTOR ON MOUSE MACROPHAGES BINDING IgG<sub>3</sub>\*

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Among the effector functions exhibited by immunoglobulins is the ability to bind to Fc receptors on macrophages, lymphocytes, and granulocytes (1–10). It has recently become evident that there are different types of Fc receptors on different types of cells (11) and, moreover, that the same cell may express more than one type of Fc receptor (1, 2, 5). For example, mouse macrophage Fc receptors do not bind IgM, whereas some lymphocyte Fc receptors do (7). Mouse macrophages are known to express at least two different Fc receptors, one specifically binding mouse IgG<sub>1</sub> and IgG<sub>2b</sub>, the other binding IgG<sub>2a</sub> (12, 13). The Fc receptors are distinguishable by certain other properties as well. The IgG<sub>2a</sub> receptor is sensitive to trypsin and shows decreased binding at 4°C and in the presence of cytochalasin B; the IgG<sub>1</sub>-IgG<sub>2b</sub> receptor is trypsin resistant and unaltered at 4°C by cytochalasin B (1, 13).

The IgG<sub>3</sub> class of mouse immunoglobulins has not been studied extensively with respect to its binding to Fc receptors. IgG<sub>3</sub> has an Fc fragment that is serologically and structurally different from IgG<sub>1</sub> and IgG<sub>2</sub> (14). Functionally, it has been shown to have an increased ability to cross the placenta (14). Earlier experiments have shown that monomeric IgG<sub>3</sub> does not inhibit the binding of monomeric IgG<sub>2a</sub> to its Fc receptor (14). These and other early results have led to the assumption that IgG<sub>3</sub> does not bind to Fc receptors. In light of the evidence for multiple Fc receptors, we felt it necessary to examine again the binding of IgG<sub>3</sub> to Fc receptors.

We generated a monoclonal IgG<sub>3</sub> anti-sheep erythrocyte (SRBC)<sup>1</sup> antibody from a mouse spleen-mouse myeloma fusion in order to study directly the binding of IgG<sub>3</sub> to macrophage Fc receptors. Monoclonal antibodies are especially useful in such studies because they are homogeneous, they are the only mouse immunoglobulin in culture medium from the hybridoma cultures, and when reacted with their antigen, they form antigen-antibody complexes more natural for binding studies than artificial aggregates. Using this monoclonal IgG<sub>3</sub> anti-SRBC antibody, we found that IgG<sub>3</sub> does bind to macrophages through a third, independent IgG Fc receptor.

## Materials and Methods

*Cells.* J774 is a reticulum cell sarcoma from a BALB/c mouse with macrophagelike properties that has been adapted to culture (15). J774.2 is a clone from the tissue culture line.

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<sup>1</sup> Abbreviations used in this paper: BDB, bis-diazotized benzidine; PBS, phosphate-buffered saline; SRBC, sheep erythrocytes.

A series of subclones of J774.2 were picked and screened for their ability to bind IgG<sub>3</sub>. J774.2.1 was found to lack IgG<sub>3</sub> receptors. It has grown to mass culture and recloned to produce J774.2.1.4. P388D1 is a macrophagelike cell line with Fc receptors and phagocytic ability. FC1.4 and FC1.6 are subclones of the FC1 cell line which arose during a fusion of MPC 11 myeloma cells to spleen cells from a BALB/c mouse immunized with SRBC. FC1.4 and FC1.6 were selected for their inability to phagocytize IgG<sub>2a</sub>-SRBC.

Primary macrophages were obtained from resident peritoneal cells and from peritoneal cells 4 d after an intraperitoneal injection of thioglycolate broth (Difco Laboratories, Detroit, Mich.). The method for isolating adherent cells has been described previously (1).

*Myeloma Proteins.* Myeloma proteins were obtained by injecting 10<sup>7</sup> MOPC21 (IgG<sub>1</sub>), MPC11 (IgG<sub>2b</sub>), MOPC173 (IgG<sub>2a</sub>), or J606 (IgG<sub>3</sub>) cells into the peritoneal cavity of pristinely primed BALB/c mice (16). The ascites fluid was precipitated with 50% saturated ammonium sulfate. A purified  $\gamma$ -globulin fraction was obtained by DEAE chromatography (1). It was determined by agarose gel electrophoresis and by Ouchterlony analysis that no fraction had any contaminating protein of another subclass. Flopc 21 was purchased from Bionetics, Kensington, Md. Monomeric protein was prepared by centrifuging the protein for 30 min at 150,000 g.

*Preparation of Ig-coated SRBC.* The methods of Bianco et al. (17) were used. SRBC were incubated with antibody for 30 min at 37°C, washed, and resuspended to 0.5%. The antibodies were obtained from cloned hybridoma lines making anti-SRBC antibody. 5  $\mu$ l of ascites fluid was incubated with 1 ml of a 5% solution of SRBC.

The IgG<sub>3</sub>-producing line was derived from a fusion of drug-marked P<sub>3</sub> cells with spleen cells from a BALB/c mouse immunized with SRBC. Segregants no longer making the myeloma IgG<sub>1</sub> heavy chain were selected. The IgG<sub>3</sub> antibody was identified by Ouchterlony analysis with commercial anti-IgG<sub>3</sub> (Meloy Laboratories, Inc., Springfield, Va.) and with antiserum raised in the laboratory of Dr. John Cebra, University of Pennsylvania, against J606 protein and absorbed with IgG<sub>1</sub>, IgG<sub>2a</sub>, and IgG<sub>2b</sub>.

*Fc Rosettes and Fc-mediated Phagocytosis.* This was done as previously described (1). Cells adhered to glass coverslips were incubated for 30 min at 37° or 4°C, washed, and assayed for rosettes. Attachment of three or more SRBC signified a rosette. For phagocytosis, the cells were incubated for 1 h at 37°C, free SRBC were lysed in hypotonic solution, and intracellular SRBC were assayed. A phagocytic cell was any cell with three or more ingested SRBC. Controls were run with SRBC incubated in normal mouse serum, and <5% of cells rosetted or phagocytized. To study the effect of trypsin and cytochalasin, macrophages were incubated in 1 mg/ml of crystallized trypsin in phosphate-buffered saline (PBS), or 10  $\mu$ g/ml of cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in PBS for 30 min at 37°C, washed, and assayed for rosetting ability.

*Inhibition of Binding by Myeloma Proteins.* MPC11 (IgG<sub>2b</sub>), MOPC173 (IgG<sub>2a</sub>), MOPC21 (IgG<sub>1</sub>), J606 (IgG<sub>3</sub>), or Flopc 21 (IgG<sub>3</sub>) protein was aggregated with bis-diazotized benzidine (BDB) (18). Macrophages were pretreated for 5 min with the myeloma protein in PBS and then Ig-SRBC were added. Rosettes were assayed at 20 min.

*Inhibition of Binding by Protein A.* 20  $\mu$ l of a 5% solution of SRBC were incubated with 50  $\mu$ l of protein A at 5 mg/ml and 200  $\mu$ l of either IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, or IgG<sub>3</sub> anti-SRBC antibody. The titer of the anti-SRBC antibody in each case was 1:500 using indirect hemagglutination with an anti- $\kappa$  antibody. The SRBC were washed and assayed for rosette formation.

## Results

IgG<sub>3</sub>-coated SRBC formed rosettes with primary resident and thioglycolate-induced peritoneal macrophages, and with J774.2 and P388D1 cell lines (Table I).

In competitive inhibition experiments (Tables I and II), aggregates of IgG<sub>1</sub>, IgG<sub>2a</sub>, and IgG<sub>2b</sub> all failed to inhibit rosetting of IgG<sub>3</sub>-coated SRBC, suggesting a separate receptor for IgG<sub>3</sub>. In contrast, two IgG<sub>3</sub> myeloma proteins, Flopc 21 and J606, did inhibit rosetting when aggregated chemically with BDB. These results imply that the

TABLE I  
*Competition with Other IgG Subclasses for Fc Rosetting*

	Inhibitor	J774.2	P388D1	Primary resident	Primary TG induced
IgG <sub>3</sub> -SRBC	—	97	93	87	89
	IgG <sub>1</sub> aggregated	96	93	89	89
	IgG <sub>2a</sub> aggregated	97	91	85	91
	IgG <sub>2b</sub> aggregated	98	91	86	84
	IgG <sub>3</sub> aggregated	21	22	19	22

TABLE II  
*Rosetting of IgG<sub>3</sub>-SRBC to Macrophages*

	Inhibitor*	J774.2	P388D1	J774.2.1.4	Primary resident	Primary TG induced
IgG <sub>3</sub> -SRBC	—	98	98	2	87	93
	J606 aggregated	17	21	2	17	21
	Flopc 21 aggregated	19	20	4	22	19
	Flopc monomer	95	91	3	84	97

\* Inhibiting proteins were used at a concentration of 100 µg/ml.

TABLE III  
*Protein A Inhibition of Fc-mediated Rosettes*

	J774.2
IgG <sub>3</sub> alone	98
IgG <sub>3</sub> + protein A	21
IgG <sub>1</sub> alone	97
IgG <sub>1</sub> + protein A	88
IgG <sub>2a</sub> alone	99
IgG <sub>2a</sub> + protein A	18
IgG <sub>2b</sub> alone	99
IgG <sub>2b</sub> + protein A	21

IgG<sub>3</sub> antibody is binding to the macrophage through its Fc terminal. Monomeric IgG<sub>3</sub> did not inhibit rosetting.

The rosetting of IgG<sub>3</sub>-SRBC was unaffected at 4°C, by cytochalasin B, or by trypsinization of the macrophages. In this respect, the IgG<sub>3</sub> receptor behaves like the receptor for IgG<sub>1</sub> and IgG<sub>2b</sub> and unlike the receptor for IgG<sub>2a</sub>, which is altered at 4°C, and by both cytochalasin and trypsin.

Although inhibition of rosetting by aggregated IgG<sub>3</sub> suggested that the binding of antibody-coated SRBC was Fc mediated, we wanted to show by a second method that IgG<sub>3</sub> was bound to receptors on macrophages through its Fc portion. We therefore examined whether protein A could inhibit the binding of IgG<sub>3</sub>. Protein A will bind to the Fc portion of several immunoglobulins, including IgG<sub>3</sub> and IgG<sub>2</sub>. It binds poorly to IgG<sub>1</sub> (19). It has been further shown that protein A will compete with Fc receptors for Fc binding (20). When protein A was incubated with IgG<sub>3</sub>-coated SRBC, it inhibited rosette formation. As expected, it also inhibited rosetting of IgG<sub>2</sub>-coated SRBC and not of IgG<sub>1</sub>-coated SRBC (Table III).

J774.2.1.4 is a randomly picked subclone of J774.2. It did not rosette IgG<sub>3</sub>-SRBC

TABLE IV  
*Phagocytosis of IgG-SRBC*

	J774.2	P388D1	J774.2.1.1	Primary resident	Primary TG induced
IgG <sub>3</sub>	89	87	2	83	91
IgG <sub>1</sub>	91	90	88	85	89
IgG <sub>2a</sub>	93	88	91	91	92
IgG <sub>2b</sub>	92	87	94	85	92

TABLE V  
*Phagocytosis by Variant Lines*

	FC1.4	FC1.6
IgG <sub>1</sub>	89	92
IgG <sub>2a</sub>	14	16
IgG <sub>2b</sub>	91	93
IgG <sub>3</sub>	90	90

at all. It did, however, rosette IgG<sub>1</sub>-, IgG<sub>2a</sub>-, and IgG<sub>2b</sub>-coated SRBC in a manner indistinguishable from the parent cell line (Tables II and IV). These data again suggest that the receptor for IgG<sub>3</sub> is independent of the other IgG receptors.

We have previously reported variants from the FC1 macrophage cell line that have altered phagocytosis through the IgG<sub>2a</sub> receptor (1). These variant lines with a selective defect in phagocytosis were both able to phagocytize IgG<sub>3</sub>-coated SRBC, suggesting again that the IgG<sub>3</sub> receptor is independent of the IgG<sub>2a</sub> receptor (Table V).

### Discussion

It has previously been thought that IgG<sub>3</sub> does not bind to Fc receptors on mouse macrophages. This was based on data showing the inability of IgG<sub>3</sub> to compete successfully with IgG<sub>2a</sub> for Fc binding (14). It has since become clear that macrophages possess more than one Fc receptor and that each receptor has a restricted specificity (1, 5, 12). Two receptors have been clearly defined: one for IgG<sub>1</sub> and IgG<sub>2b</sub>, and the other for IgG<sub>2a</sub> (4, 13).

The studies reported here indicate that there is also a separate Fc receptor on mouse macrophages for IgG<sub>3</sub>. This Fc receptor is present on resident peritoneal macrophages as well as on thioglycolate-induced macrophages. In addition, the J774.2 cell line and the P388D1 cell line exhibit this receptor. This receptor is independent from the two previously identified receptors because (a) the other IgG subclasses do not compete with IgG<sub>3</sub> for binding; (b) J774.2.1.4, a subclone of J774.2, is unable to bind or phagocytize IgG<sub>3</sub> complexes but is unaffected in its IgG<sub>2a</sub> and IgG<sub>1</sub>-IgG<sub>2b</sub> receptors; and, (c) variants of the FC1 line that are altered in their ability to phagocytize through the IgG<sub>2a</sub> receptor have an intact ability to phagocytize IgG<sub>3</sub>-SRBC.

The IgG<sub>3</sub> Fc receptor is similar to the receptor for IgG<sub>1</sub> and IgG<sub>2b</sub> in that it is not affected at 4°C or by cytochalasin B or trypsin. It resembles the other IgG receptors in its ability to mediate phagocytosis. In addition, we have reported that IgG<sub>3</sub> can also mediate antibody-dependent cell-mediated cytotoxicity of SRBC.

That IgG<sub>3</sub> should bind through a separate receptor is not surprising, because structurally and serologically it is the most dissimilar of the IgG subclasses (14). The identification of this third receptor, however, raises again the question of why several independent Fc receptors are present on macrophages. On macrophages, each receptor is capable of mediating both phagocytosis and antibody-dependent cell-mediated cytotoxicity (21). Other cell types that bear Fc receptors may possess only one or two of these receptors in order to restrict their response to antibody or immune complexes (8, 22, 23). If Fc receptors on other cell types have the same specificity as on macrophages, perhaps the multiplicity of receptors allows for antibody-specific responsiveness in other cell types. The multiplicity may be a consequence of the amount of information that must be carried in the constant region of the immunoglobulin molecule; the constraints of subclass specificity may make separate Fc receptors necessary. Further studies on both the biologically active sites of the IgG molecules and the nature of Fc receptors on other cell types are necessary to resolve these questions.

### Summary

Monoclonal antibodies to sheep erythrocytes (SRBC) have proved useful in identifying two Fc receptors on mouse macrophages, one for IgG<sub>2a</sub>, and one for IgG<sub>1</sub> and IgG<sub>2b</sub>. We have used monoclonal IgG<sub>3</sub> anti-SRBC to identify a third Fc receptor on mouse macrophages which binds IgG<sub>3</sub> uniquely. This receptor is present on primary resident and thioglycolate-induced peritoneal macrophages and on some macrophage cell lines. The binding of IgG<sub>3</sub>-coated SRBC is inhibited by aggregated but not monomeric IgG<sub>3</sub>, and not by IgG<sub>1</sub>, IgG<sub>2a</sub>, and IgG<sub>2b</sub> aggregates. It is unaffected by treating the macrophages with trypsin or cytochalasin B and occurs at both 4° and 37°C. IgG<sub>3</sub>, like all other IgG subclasses, mediates phagocytosis. We have also generated a variant macrophage line which bears the receptors for IgG<sub>1</sub> and IgG<sub>2b</sub> and for IgG<sub>2a</sub>, but not for IgG<sub>3</sub>.

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