SITE OF BINDING OF MOUSE IgG2b TO THE Fc RECEPTOR ON MOUSE MACROPHAGES*

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Immunoglobulins bind through their Fc regions to specific receptors on macrophages. On mouse macrophages, there are at least two such Fc receptors: one which binds the mouse IgG2a subclass and a second which binds mouse IgG2b and probably IgG1 as well (1-4). Recently, we have used pure preparations of homogeneous (hybridoma) anti-sheep erythrocytes (SRBC) antibodies to confirm the presence and subclass specificity of the macrophage receptors for IgG2a and IgG2b (3). That these two receptors are discrete has also been shown by isolating variant macrophage cell lines which either lack or have alterations in one of these two Fc receptors (2, 3).

In the experiments to be described in this paper, we have used immunoglobulins with heavy-chain deletions or recombinations, produced by variants of the MPC 11, IgG2b-producing, mouse myeloma cell line to determine the portion of the Fc region of IgG2b which binds to the mouse Fc receptors. With these variants we have shown that the mouse macrophage Fc receptor for IgG2b recognizes a site(s) in the C_H2 domain of the IgG2b molecule.

Materials and Methods

Cells. The use of J774.2, FC-1, and peritoneal macrophages to study macrophage Fc receptors has been described previously (3). J774 is a mouse reticulum cell sarcoma with macrophage-like properties which was adapted to culture by repeated passage between BALB/c mice and tissue culture. J774.2 is a subclone of the tissue culture line which stably secretes ≈10 μg of lysozyme/10^6 cells per 24 h. FC-1 is a macrophage-like cell line which arose during the fusion of a thioguanine-resistant subclone (45.6 TG1.7) (3) of the MPC 11, IgG2b, κ-producing mouse myeloma cell line to spleen cells from a BALB/c mouse immunized with SRBC.

Peritoneal cells were obtained from untreated BALB/c mice as previously described (3). Tissue culture cells were maintained in Petri dishes (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) in Dulbecco's modified Eagle's medium (H-21) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with penicillin, streptomycin, glutamine, nonessential amino acids, 20% heat-inactivated horse serum (Flow Laboratories Inc., Rockville, Md.), and 10% NCTC109 (Microbiological Associates, Walkerville, Md.).

Myeloma Proteins. Approximately 10^7 cells were injected into the peritoneal cavity of Pristane (2,6,10,14-Tetramethylpentadecane, Aldrich Chemical Co., Inc. Milwaukee, Wis.) treated BALB/c mice (5). The ascites fluid which formed was removed, separated from the cells, and precipitated with 50% saturated ammonium sulfate. Purified immunoglobulins from MPC 11 and MOPC 173 were obtained by two steps of ion-exchange chromatography (3). Variant proteins were also purified by ion-exchange chromatography using an alternate buffer system (6). Purified proteins were examined for contaminating proteins of another subclass by agarose
gel electrophoresis (7) and by Ouchterlony analysis with commercial subclass-specific antisera (Meloy Laboratories Inc., Springfield, Va.) or with our own antisera which have been made subclass specific on immunoadsorbant columns. The preparations used in the studies reported below contained no detectable contamination with other subclasses.

The Fc fragments of MPC 11, ICR 11.19.3, and ICR 9.9.2.1 were isolated as previously described (6).

Preparation of Ig-coated SRBC. The methods described by Bianco et al. were used (8). The mouse antisera were from multiply recloned anti-SRBC hybridomas which no longer make the myeloma heavy chain.

Fc Rosettes. Glass coverslips were sterilized and placed in 25-cm² Petri dishes. Approximately 5 × 10⁶ cells were added in 5 ml of medium and incubated overnight. To assay rosettes, coverslips were removed, covered with Ig-coated SRBC, and incubated at 37°C for 30 min. They were then rinsed in Hanks' balanced salt solution and 100 cells were assayed for the presence of rosettes. Attachment of three or more SRBC signified a rosette.

Purified proteins were used to inhibit rosette formation at a concentration of 100 μg/ml in phosphate-buffered saline, aggregated either with bisdiazotized benzidine (BDB) (9) or with 10 μl of a rabbit anti-mouse κ-chain antiserum. The optimal amount of anti-κ antiserum was determined by titrating it with a fixed amount of myeloma protein and selecting the antigen-antibody mixture which inhibited rosetting of antibody-coated SRBC. Macrophages were pretreated for 15 min with purified myeloma protein after which Ig-coated SRBC were added. After 30 min, cells were examined for rosette formation.

Results

Controls for the Binding of Antibody-coated SRBC by Macrophages. SRBC coated with either monoclonal IgG₂b or IgG₂a anti-SRBC antibody will rosette with Fc receptors on two macrophage cell lines, J774.2 and FC1, and on primary peritoneal macrophages (3). As shown previously, rosetting of IgG₂a-coated SRBC was inhibited by aggregated MOPC 173 (IgG₂a) protein and not by aggregated MPC 11 (IgG₂b) protein, whereas rosetting of IgG₂b-coated SRBC was inhibited by aggregated MPC 11 protein but not by MOPC 173 protein (Table I A). As a further control for the specificity of the Fc receptors, ICR 9.9.2.1 was used. This variant immunoglobulin contains the idiotype of the parental MPC 11 protein (7), has a normal molecular weight, and the sequence of its Fc region is that of IgG₂a (6). It inhibited the binding of IgG₂b-coated SRBC and not of IgG₂a-coated SRBC (Table I A). This showed that a variant immunoglobulin would behave as predicted from the primary sequence of its Fc region.

Binding of M 311. M 311, a variant having a short heavy chain with a deleted C₃ domain (10, 11, and A. Kenter and B. K. Birshtein, unpublished data), inhibited binding of IgG₂b-coated SRBC but not of IgG₂a-coated SRBC (Table I A). This result suggested that an intact C₃ domain is not required for IgG₂b to bind to the Fc receptor. Because this was our first indication that the C₃ domain might be involved in binding, we wanted to rule out the possibility that BDB treatment had exposed previously hidden and perhaps nonphysiologic Fc binding sites on the M 311 protein. Therefore, M 311 was independently aggregated with rabbit anti-mouse κ-chain antibody. The antibody-aggregated protein also inhibited rosette formation and again showed IgG₂b subclass specificity (Table I B).

Binding of ICR 11.19.3. The variant protein produced by ICR 11.19.3 contains the MPC 11 idiotype (7), IgG₂b sequences in the N-terminal portion of the heavy chain, and IgG₂a sequences throughout its C₃ domain (12). The cross-over point between IgG₂b and IgG₂a sequences seems to lie in the C-terminal segment of the C₃ domain, (B. K. Birshtein, R. Campbell, and M. L. Greenberg, manuscript in preparation). When aggregated, this protein inhibited rosette formation with both IgG₂b- and IgG₂a-coated SRBC (Table I A). The inhibition was, however, less complete for each Fc receptor than with the appropriate normal immunoglobulin.
Table I

| Cell line | Inhibitor | IgGzb-SRBC | IgGzb-SRBC |
|-----------|-----------|------------|------------|
|           |           | Rosettes   | Inhibition |
|           |           | %          | %          |
| A         | FC1       | 98         | 100        |
|           | MPC 11    | 7          | 94         |
|           | MOPC 173  | 88         | 11         |
|           | ICR 9.9.2.1 | 90      | 10         |
|           | M 311     | 5          | 95         |
|           | ICR 11.19.3 | 34      | 66         |
| J774.2    |           | 97         | 97         |
|           | MPC 11    | 11         | 89         |
|           | MOPC 173  | 88         | 10         |
|           | ICR 9.9.2.1 | 88      | 10         |
|           | M 311     | 7          | 93         |
|           | ICR 11.19.3 | 29      | 70         |
| 1°        |           | 88         | 87         |
|           | MPC 11    | 9          | 90         |
|           | MOPC 173  | 86         | 8          |
|           | ICR 9.9.2.1 | 83      | 7          |
|           | M 311     | 6          | 94         |
|           | ICR 11.19.3 | 26      | 71         |
| B         | FC1       | 97         | 100        |
|           | MPC 11    | 13         | 87         |
|           | MOPC 173  | 89         | 9          |
|           | M 311 (BDB aggregated) | 5  | 95         |
|           | M 311 (rabbit anti-K) | 8  | 92         |
| C         | FC1       | 100        | 99         |
|           | ICR 9.9.2.1 Fc | 95  | 5          |
|           | ICR 11.19.3 Fc | 33  | 67         |
|           | MPC 11 Fc | 33         | 67         |

Binding of Fc Fragments. Aggregated Fc fragments of MPC 11, ICR 9.9.2.1, and ICR 11.19.3 inhibited rosette formation in the same way as the intact molecules (Table I C). This result implies that neither the Fab fragment nor the CH1 domain are required to stabilize the Fc fragment for binding to an Fc receptor.

Discussion

The constant region of the IgG molecule contains three domains of ~110 residues each. These domains are homologous in amino acid sequence to each other and to the corresponding domains of other immunoglobulin classes. The sequences of mouse IgG2a and IgG2b constant regions are of special interest to the studies reported here. The complete sequence of the IgG2a constant region was determined by Fougereau and colleagues (13). Recently we have reported partial amino acid sequences in the IgG2b Fc region (6). The C1-2 domains of mouse IgG2a and IgG2b are very similar because of the 66 residues determined, only 8 are different. The C2-3 domain shows more differences; in the C-terminal 34 residues alone, IgG2b and IgG2a differ in 20 positions.

Each domain is folded into a relatively independent tertiary structure which has been postulated by Edelman and Gall to be responsible for different functions of the immunoglobulin molecule (14). The aim of these studies was to use variant myeloma proteins to try to determine which parts of the mouse heavy chain react with the Fc receptors on mouse macrophages. Whereas alterations or deletions in these variant
molecules may cause a change in folding, it was hoped that the use of the intact proteins would minimize the possibility both of exposing new sites for macrophage binding and of obscuring sites that require stabilization from adjacent portions of the molecule.

We used three homogeneous myeloma proteins with altered heavy chains to inhibit the binding of monoclonal antibody-antigen complexes to Fc receptors on two mouse macrophage cell lines and on primary peritoneal macrophages. The ICR 9.9.2.1 protein, which appears identical in its Fc region to the IgG2a protein, MOPC 173(6) and bound only to the IgG2a Fc receptor, served as an internal control to demonstrate that a variant immunoglobulin would display the subclass specificity expected from its linear sequence. The two other variant proteins, M 311 and ICR 11.19.3, bound specifically to the IgG2b Fc receptor. Knowledge of their sequences allows us to conclude that the C\textsubscript{H}2 domain is involved in the binding of mouse IgG2b to mouse macrophage Fc receptors because in both variants the C\textsubscript{H}3 domain of IgG2b is missing: M 311 totally lacks any C\textsubscript{H}3 domain (10, 11, and A. Kenter and B. K. Birshstein, unpublished observations) and ICR 11.19.3 has a C\textsubscript{H}3 domain that is entirely IgG2a-like (B. K. Birshstein, R. Campbell, and M. L. Greenberg, manuscript in preparation).

The recombinant molecule, ICR 11.19.3, is less effective than either normal IgG2b or M 311 in binding to the IgG2b Fc receptor. This finding could be explained if the cross-over has occurred within the sequence(s) that makes contact with the IgG2b receptor or if the contact residues are all present but have changed in their tertiary structure, availability, or stability because of new neighboring sequences. The recombinant molecule also specifically inhibited the binding of IgG2b to the IgG2b receptor, but again this inhibition was partial and the same possible explanations exist. The data do not prove domain specificity for the binding of IgG2b but do suggest that binding can occur through some sequences located between the terminal portion of the C\textsubscript{H}2 domain and the end of the C\textsubscript{H}3 domain.

Our results therefore implicate the C\textsubscript{H}2 domain in the binding of at least one subclass of mouse immunoglobulin to mouse macrophage receptors. Some previous studies have shown a similar result. For example, Huber et al. have found Fc receptor binding activity in the F(ab')\textsubscript{2} fragments of human IgG (15). In the guinea pig, Alexander et al. (16) studied the binding of IgG2 to peritoneal macrophages. They found that an intact C\textsubscript{H}2 domain was required for cytophilic binding and that the interchain disulfide bonds stabilized the binding site. Ovary et al. found that the fragment of rabbit IgG lacking the C\textsubscript{H}3 domain (F\textsubscript{ab}) binds to guinea pig lung macrophages whereas the C\textsubscript{H}3 fragment alone does not (17).

However, many other studies which have used heterologous systems, heterogeneous antibodies and a variety of assays, have implicated the C\textsubscript{H}3 domain. For example, Yasmeen et al. (18) using a heterologous system, found that human IgG\textsubscript{1} bound to the Fc receptor of guinea pig macrophages through the C\textsubscript{H}3 domain. They showed that C\textsubscript{H}3-coated SRBC could bind to macrophages; in addition, they could inhibit the binding of whole protein to macrophages with unaggregated C\textsubscript{H}3 fragments. Okafor et al. (19) used a homologous system in which they studied the binding of unfraccionated human IgG to human monocytes. They tested the C\textsubscript{H}3 fragment of each IgG subclass for its ability to inhibit the binding of whole, unfraccionated IgG-coated RBC and found that the C\textsubscript{H}3 fragments of IgG\textsubscript{1} and IgG\textsubscript{3} were effective although not so effective as unfraccionated IgG.

Dissanayake and Hay have studied the question of Fc binding using mouse myeloma proteins and mouse macrophages. They found direct binding of unaggre-
gated IgG1, IgG2a, and IgG2b, and of the unaggregated C\textsubscript{H}3 domains of IgG1 and IgG2b to macrophages (20). However, other results (1, 21) in the mouse systems suggest that at least some of the IgG subclasses will not bind to Fc receptors unless aggregated.

It is possible that Fc receptors on various cell types differ in their specificity in binding. Ramasamy et al. (22) were unable to inhibit rosette formation of heterogeneous Ig-coated RBC to mouse lymphocytes with a mouse IgG1 immunoglobulin missing the C\textsubscript{H}3 domain. They therefore suggested a site in the C\textsubscript{H}3 domain was responsible for the binding of IgG1 to lymphocytes. On mouse macrophages, a single Fc receptor seems to recognize both IgG1 and IgG2b, and the studies reported here show that a site in the C\textsubscript{H}2 domain is responsible for the binding of IgG2b to this receptor. Consequently, either IgG1 and IgG2b bind to a single Fc receptor through different domains or Fc receptors on lymphocytes differ from those on macrophages. Fc receptors on other cell types and in different species might also differ. For example, experiments by McNabb et al. showed that human IgG binds to macrophages through the C\textsubscript{H}3 domain although it binds to Fc receptors on human placental tissue through sites in both the C\textsubscript{H}2 and C\textsubscript{H}3 domains (23). In rabbits, however, Tsay and Schlamowitz have shown that rabbit IgG binds to rabbit yolk sac membrane through the C\textsubscript{H}2 domain (24).

These differing results suggest that different subclasses of IgG may have different domains for binding to Fc receptors. Different cell types may have Fc receptors with different specificities. Different species may have the sequences responsible for certain functions localized in different domains.

The experiments reported in this paper use a homologous murine system with homogeneous reagents to show the binding of the C\textsubscript{H}2 domain of IgG2b to macrophage Fc receptors. These results also demonstrate the potential usefulness of variant immunoglobulins in probing the determinants responsible for the effector functions of immunoglobulins. The combined use of homogeneous antibodies and such variant proteins should make it possible to compare the specificity of Fc receptors on a variety of cell types.

**Summary**

Three mouse immunoglobulins with altered heavy chains have been used to study the specificity of the mouse IgG2a Fc receptor on mouse macrophages. These immunoglobulins were synthesized by variant clones derived from the MPC 11, IgG2b-producing mouse myeloma cell line. One variant, whose Fc seems identical to that of an IgG2a myeloma protein of known sequence, binds specifically to the IgG2a Fc receptor. A second variant, which makes a short heavy chain lacking the C\textsubscript{H}3 domain, binds specifically to the IgG2b Fc receptor. The third variant makes a hybrid IgG2b-IgG2a heavy chain whose C\textsubscript{H}3 domain is entirely IgG2a-like and binds to both IgG2a and IgG2b Fc receptors. These data suggest that the binding of mouse IgG2b immunoglobulins to the mouse macrophage Fc receptor involves a site within the C\textsubscript{H}2 domain and indicate that immunoglobulins with altered heavy chains are a useful tool to probe Fc receptors.

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