STRUCTURAL EVIDENCE FOR DISTINCT IgG SUBCLASS-SPECIFIC Fc RECEPTORS ON MOUSE PERITONEAL MACROPHAGES*

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Receptors that recognize and bind the Fc portion of IgG have been identified on a wide variety of cells, including macrophages, lymphocytes, and polymorphonuclear leukocytes. These receptors function in the phagocytosis (1) and cytolysis (2) of IgG-coated target cells, and an increasing body of evidence indicates that they also have an incompletely characterized immunoregulatory role (3). Fc receptors that are found on different types of cells express different affinities for subclasses of IgG, and Fc receptors that bind other classes of immunoglobulin (IgM and IgE) have been described (4, 5). With the exception of the IgE receptor found on mast cells and basophils, the functional significance of immunoglobulin class and subclass-specific Fc receptors remains largely unexplained.

Mouse macrophages and macrophage-like cell lines appear to possess two Fc receptors (FcR)[1] that can be distinguished by their specificity for different mouse IgG subclasses and their protease sensitivity (6–11). These studies indicate that mouse macrophages have a protease-sensitive FcR that binds monomeric and aggregated IgG2a and a protease-resistant FcR that binds aggregates of IgG1 and IgG2b. Identification of these distinct FcR has primarily relied upon binding studies using intact cells, but at the present time there is no structural evidence to confirm the existence of discrete Fc-binding molecules. In this paper we report on the isolation and characterization of two discrete Fc-binding proteins from mouse peritoneal macrophages. The two proteins exhibit different IgG subclass-binding specificity and trypsin sensitivity, and appear to represent the IgG subclass-specific FcR present on mouse macrophages.

Materials and Methods

Macrophages. 10- to 14-wk-old female C57BL/6 mice were injected intraperitoneally with 1 ml of brewers' thioglycollate medium (Difco Laboratories, Detroit, Mich.) and cells were

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[1] *Abbreviations used in this paper:* CNBr, cyanogen bromide; E, uncoated SRBC; EA, SRBC sensitized with rabbit IgG anti-SRBC; E2a, monoclonal IgG2a anti-SRBC; E2b, monoclonal IgG2b anti-SRBC; FcR, Fc receptor(s); FCS, fetal calf serum; IEF, isoelectric focusing; MEM, minimal essential medium; OA, ovalbumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes.

harvested from the peritoneal cavity 4 d later. To obtain macrophages, 1.5–2 × 10^7 peritoneal exudate cells in 2 ml of minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (MEM-FCS) (Flow Laboratories, Inc., Rockville, Md.) were incubated in 60-mm plastic tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 1 h in a 95% air-5% CO_2 atmosphere at 37°C. Nonadherent cells were removed by four successive washes with phosphate-buffered saline (PBS). The purity of the adherent cell monolayer was determined by staining for nonspecific esterase activity (12) and phagocytosis of latex beads (13).

Myeloma Proteins and IgG Proteins. The following purified murine myeloma proteins were obtained from Litton Bionetics, Inc., Kensington, Md., or purified from clarified ascites fluid by a combination of $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography on DEAE-52 (Whatman, Inc., Clifton, N. J.): UPC 10 and RPC 5 (IgG2a), MOPC 195 and MOPC 141 (IgG2b), MOPC 21 (IgG1), and J606 (IgG3). Purified proteins were examined for contaminating proteins of other IgG subclasses by double diffusion and immunoelectrophoretic analysis with IgG subclass-specific antisera (Miles Laboratories, Inc., Elkhart, Ind.). No detectable contamination of the IgG2a and IgG1 proteins was noted. Slight reactivity of MOPC 141 (IgG2b) was found with anti-IgG2a, however, by isoelectric focusing, all the proteins appeared essentially pure. Rabbit IgG anti-ovalbumin (OA) and $F(ab')_2$ anti-OA were obtained from N. L. Cappel Laboratories, Inc., Cochranville, Pa. Human IgG was obtained from Miles Laboratories, and $F(ab')_2$ fragments were prepared by pepsin digestion and fractionation on Sephadex G-150 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) as previously described (14). The absence of contaminating intact IgG or Fc determinants in the $F(ab')_2$ preparations was confirmed by immunodiffusion and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Binding of IgG-sensitized Sheep Erythrocytes. Sheep erythrocytes (SRBC) were sensitized with a nonhemagglutinating titer of rabbit IgG anti-SRBC (lot 50767; Cordis Laboratories Inc., Miami, Fla.). Monoclonal IgG2a (UN-2) anti-SRBC and monoclonal IgG2b (U88) anti-SRBC were the gifts of Dr. Betty Diamond, Albert Einstein Medical School, Bronx, N. Y., and were used at a nonhemagglutinating titer to opsonize the SRBC. Peritoneal exudate cells were suspended to 5 × 10^6 cells/ml in MEM-FCS and 100 μl was added to each well of an eight-chamber Lab-Tek tissue culture slide (Arthur H. Thomas Co., Philadelphia, Pa.). Sensitized SRBC were added to the adherent cells and after a 30-min incubation at 37°C the wells were washed three times with PBS to remove the unbound SRBC. Rosette formation was scored by determining the percentage of macrophages with three or more attached erythrocytes. At least 200 macrophages were counted per well, and all experiments included control wells with uncoated SRBC. To determine the effect of trypsin treatment on rosette formation, some wells were pretreated with trypsin (Worthington Biochemical Corp., Freehold, N. J.) at a final concentration of 1 mg/ml in PBS for 30 min at 37°C. After three washes, the SRBC were added as described above. The effect of different subclass myeloma proteins upon rosette formation was determined by preincubating the macrophages with different quantities of heat-aggregated proteins for 15 min at 37°C before adding SRBC.

Surface Radioiodination and Lysis of Cells. Peritoneal exudate cells were iodinated using carrier-free [^{125}I]Na (Amersham Corp., Arlington Heights, Ill.) and 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, Ill.) as previously described (15). 5 × 10^6 peritoneal exudate cells in 0.5 ml calcium-magnesium-free PBS were labeled in scintillation vials coated with 25 μg Iodogen for 10 min at ambient temperature. After the reaction was stopped by removing cells from the vial, macrophages were purified by adherence to plastic. The viability of the peritoneal exudate cells at the end of the iodination procedure was >95% as determined by the trypan blue exclusion test. To solubilize the iodinated membrane components, macrophages were scraped off the petri dishes at a concentration of 1–2 × 10^7 cells/ml in a cold lysis buffer that contained 0.5% NP-40 in 50 mM Tris-HCl (pH 7.0)-150 mM NaCl supplemented with 25 μg/ml phenylmethylsulphonyl fluoride and 0.25% ε-amino-*n*-caproic acid. To minimize proteolysis, all manipulations were carried out in the cold. The cells were vortexed gently for 30 s, and insoluble cell debris and nuclei were removed from the suspension by centrifugation at 800 *g* for 15 min.

Preparation of Affinity Beads. Proteins were coupled to cyanogen bromide (CNBr)-activated

Sepharose 4B (Pharmacia Fine Chemicals. Dehydrated CNBr-activated Sepharose 4B was washed with 1 mM HCl and suspended in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3. For coupling, the protein was dissolved in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3, and mixed with the beads for 16 h at 4°C. The coupling buffer was removed and unreacted coupling sites were blocked by incubation of the beads with 1 M ethanolamine, pH 8.6 for 2 h at 22°C. The beads were alternately washed with 0.1 M sodium borate/0.5 M NaCl, pH 8.3, and 0.1 M sodium acetate/0.5 M NaCl, pH 4.0 until the absorbance at 280 nm of the eluates was zero. The protein-coupled beads were stored in PBS/0.02% sodium azide at 4°C. Intact or $F(ab')_2$ human IgG was coupled to Sepharose 4B at 4 mg/ml of beads. Mouse myeloma proteins and human IgG and $F(ab')_2$ fragments were aggregated by heating at 63°C for 20 min before coupling. Heat-aggregated mouse myeloma proteins were coupled at 800 μg/ml of Sepharose 4B. Beads with antigen-complexed IgG or $F(ab')_2$ fragment of IgG were prepared by passing intact rabbit IgG or $F(ab')_2$ fragment anti-OA through Sepharose 4B to which OA was coupled at 4 mg/ml. The antigen-complexed beads contained 1 mg of intact IgG or $F(ab')_2$ fragment/1 ml of beads.

Isolation of FcR. All subsequent steps were performed at 4°C. The lysate from between 2×10^7 and 6×10^7 macrophages was passed repetitively through a sham affinity column that contained 0.5 ml of OA-Sepharose 4B to remove proteins that appeared to bind nonspecifically to the affinity beads. The lysate was then either divided into two portions with one portion passed through beads that contained $F(ab')_2$ fragment of IgG and the other portion passed through IgG-coupled beads, or the lysate passed sequentially through a column of $F(ab')_2$ beads and then through a column of IgG beads. The columns with rabbit and human IgG and $F(ab')_2$ fragments contained a 0.5-ml volume of beads, whereas the murine myeloma IgG columns contained 0.2 ml of beads. The beads were washed with between 10 and 40 column volumes of lysis buffer and then with two column volumes of water. Bound material was eluted by washing the beads with three to five column volumes of 0.5 N acetic acid and then with a similar amount of 0.1% SDS in 2 mM Tris-HCl buffer, pH 6.8. If the eluted material was to be analyzed by electrophoresis, it was concentrated by lyophilization.

Electrophoresis Procedures. One-dimension SDS-PAGE was performed with a vertical slab gel apparatus and the discontinuous gel system of Laemmli (16). The separating gels were 19-cm long and were prepared with a 7.5–15% linear acrylamide gradient. Samples were solubilized by heating at 100°C for 2 min in sample buffer that contained 2% SDS, 10% glycerol, 4% 2-mercaptoethanol, 0.001% bromphenol blue, and 0.0625 M Tris-HCl, pH 6.8. In some cases 2-mercaptoethanol was left out of the sample buffer.

For two-dimensional separation of proteins by charge and size, the procedure of O'Farrell (17) was used with the changes outlined below. Samples were prepared for isoelectric focusing (IEF) by heating at 100°C for 3 min in 2% SDS, 5% 2-mercaptoethanol, and 0.0625 M Tris-HCl, pH 6.8, after which the sample was lyophilized. The sample was dissolved in 15% Triton X-100, 4 M urea, and 1% Ampholines (LKB Instruments, Inc., Rockville, Md.) to yield a Triton X-100:SDS ratio of 8:1 by weight. The IEF gels contained 2% Triton X-100 in place of 2% NP-40 and 2% Ampholines (pH 3.5–10). Gels were focused 14 h at 400 V and 1 h at 500 V. Radioiodinated molecules were detected by radioautography as suggested by Laskey and Mills (18) using preexposed Kodak X-Omat R film (Eastman Kodak Co., Rochester, N. Y.) and a Rarex B Midspeed intensifying screen (GAF Corp., New York) at −70°C. The pH profile of the IEF gel was determined by measuring the surface pH of the gel at 22°C with a microcombination pH probe (model M1-410; Microelectrodes, Inc., Londonderry, N. H.). The IEF pH range of isolated proteins was approximated by comparison of the protein's position in two-dimensional gels with the pH profile of a reference IEF gel.

Results

Characterization of Mouse Peritoneal Macrophages. Peritoneal cells were harvested from mice after the intraperitoneal injection of thioglycollate. The cells were surface labeled with ^{125}I and Iodogen, and macrophages were enriched by adherence to plastic Petri dishes for 60 min at 37°C. Nonadherent cells were removed by washing the dishes, and the adherent cells characterized by staining for nonspecific esterase and ingestion of latex particles. The adherent cells were found to be between 95 and 100% esterase

and latex positive. Isolation of the macrophages by plastic adherence after surface iodination is advantageous in that cells are labeled before encountering foreign proteins, such as those contained in FCS, and only viable cells attach to the plates and are available for further study. Comparative analysis by SDS-PAGE of lysates from Iodogen and lactoperoxidase-radioiodinated cells revealed similar patterns, although a greater incorporation of ^{125}I and a higher specific activity was achieved with Iodogen. (J. Kan-Mitchell and M. S. Mitchell. Membrane proteins of peritoneal macrophages induced by different immunomodulators. Characterization of "thioglycollate broth" induced proteins. Manuscript in preparation.).

Properties of the FcR on Thioglycollate-elicited Macrophages. Macrophages that had adhered to the wells of Lab-Tek slides were incubated with uncoated SRBC (E), or SRBC sensitized with rabbit IgG anti-SRBC (EA), monoclonal IgG2a anti-SRBC (E2a), or monoclonal IgG2b anti-SRBC (E2b). In some wells the macrophages were pretreated with trypsin or heat-aggregated mouse myeloma proteins of different IgG subclasses. The results of these rosetting experiments are shown in Table I. Practically all cells (range: 91–97%) bound EA, E2a, and E2b. Trypsin treatment greatly reduced the binding of E2a, slightly reduced the binding of EA, and did not affect E2b binding. Although there was cross-inhibition of E2b binding with aggregated IgG2a, using different concentrations of myeloma proteins, the greatest inhibition was found with the same subclass of IgG. Aggregated IgG1 only inhibited E2b binding, but to

TABLE I

Effect of Trypsin and Aggregated Myeloma Proteins upon Rosette Formation by Thioglycollate-induced Peritoneal Macrophages

Pretreatment	Percent rosettes*			
	E	EA	E2a	E2b
None	3	93	97	91
Trypsin‡	1	66 (29)	30 (69)	90 (1)
Aggregated§ (0.5 mg/ml) Mouse $IgG2_a$ (0.1 mg/ml)	NT‖ NT	20 (78) NT	22 (77) 29 (70)	56 (38) 82 (10)
Aggregated§ (0.5 mg/ml) Mouse $IgG2_b$ (0.1 mg/ml)	NT NT	47 (49) NT	87 (10) 90 (7)	6 (93) 16 (82)
Aggregated§ (0.5 mg/ml) Mouse IgG1 (0.1 mg/ml)	NT NT	74 (20) NT	96 (1) 94 (3)	49 (46) 62 (32)
Aggregated§ (0.5 mg/ml) 2a and 2b	NT	6 (94)	NT	NT

* Macrophage monolayers were incubated with the following erythrocyte preparations: E, uncoated SRBC; EA, SRBC sensitized with rabbit IgG anti-SRBC; E2a, SRBC sensitized with monoclonal IgG2a anti-SRBC; E2b, SRBC sensitized with monoclonal IgG2b anti-SRBC. The percent inhibition is shown in parentheses.

‡ Macrophages were pretreated with 1 mg/ml trypsin for 30 min at 37°C and washed before the addition of erythrocytes.

§ Macrophages were pretreated for 15 min with the indicated concentration of soluble heat-aggregated myeloma proteins before the addition of erythrocytes.

‖ NT, not tested.

a lesser extent than IgG2b. These studies are consistent with the existence of a trypsin-sensitive IgG2a FcR and trypsin-resistant IgG2b/IgG1 FcR on mouse thioglycollate-elicited peritoneal macrophages. The data also indicate that in the intact cell there is either cross-competition between IgG2a and IgG2b for the two receptors, or that the binding of one IgG2 subclass sterically inhibits the binding of the other.

Fc-binding Proteins from Thioglycollate-elicited Macrophages. We prepared columns of Sepharose 4B coupled to heat-aggregated human IgG and aggregated human $F(ab')_2$ IgG or Sepharose 4B coupled to OA that had been incubated with either intact rabbit IgG anti-OA or $F(ab')_2$ anti-OA. In initial experiments, the lysates were divided and passed over the individual columns, whereas in later experiments, the entire lysate was sequentially passed over the $F(ab')_2$ column and then the IgG column. SDS-PAGE analyses of the original lysate and the acetic acid and SDS eluates from these columns are shown in Fig. 1. The acetic acid eluates from the aggregated human $F(ab')_2$ column revealed either no bands or occasional faint bands. The acetic acid eluates from the intact aggregated human IgG columns revealed two bands that were not seen in eluates from the $F(ab')_2$ column, a broad band with an ~52,000 mol wt and a narrower band with an ~67,000 mol wt. The acetic acid eluates from rabbit IgG and $F(ab')_2$ anti-OA gave the same patterns as aggregated human IgG and $F(ab')_2$ fragments, except that the quantity that was eluted was increased. The gel patterns of SDS eluates were more complicated, with several radioactive bands observed. However, as was seen with the acetic acid eluates, the profiles of SDS eluates from IgG and $F(ab')_2$ columns were identical except for the presence of the 67,000- and 52,000-dalton proteins in eluates from columns that contained intact Fc regions. The proteins that are nonspecifically bound to the columns and eluted with SDS correspond to the predominant labeled bands found in the original lysate (Fig. 1). In addition, the two Fc-binding proteins were not found in acetic acid or SDS eluates from IgG columns over which we had passed the lysates of Molt 3, an FcR-negative cell line.

When the eluates from the IgG columns were analyzed with and without the addition of 2-mercaptoethanol the two Fc-binding proteins were found in the same location on the radioautograph. Thus it appears that the two radioactive Fc-binding proteins consist of polypeptide chains that are not linked by disulfide bonds.

Separation of these proteins was obtained when the acetic acid and SDS eluates were analyzed by a combination of IEF and SDS-PAGE (Fig. 2). The findings with two-dimensional gels confirmed the results from the SDS-PAGE analysis. The acetic acid eluates from antigen-complexed IgG columns contained the two Fc-binding proteins (Fig. 2 A). The 52,000-dalton protein was clearly separated from heavy chain that eluted from the column and stained with Coomassie blue (Fig. 2 B). The acetic acid eluates from the $F(ab')_2$ column revealed Coomassie blue-stained light chain, but no heavy chain (Fig. 2 C) and no radioactive spots.

Several radioactive spots were found in the SDS eluates, but with the exception of the two Fc-specific spots, the patterns from $F(ab')_2$ and IgG columns were identical. Those radiolabeled proteins that elute with SDS and are not specifically bound by the Fc region of IgG can be reduced, but not entirely eliminated if the lysate is passed over control Sepharose 4B columns before passage over an IgG column.

There was considerable microheterogeneity in the IEF of the two Fc-binding proteins. The 67,000-dalton protein has a broad focusing range between pH 6.0 and

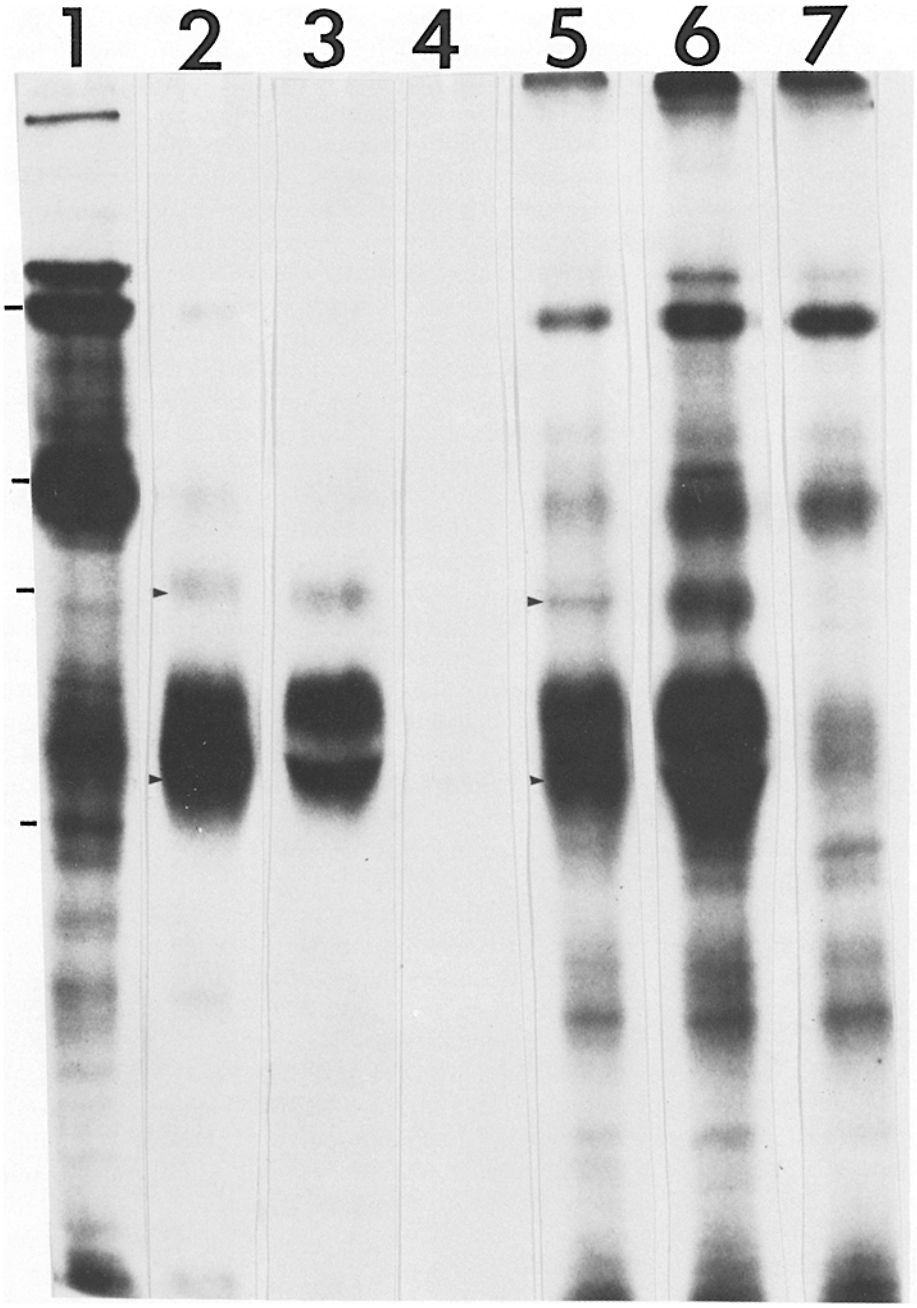

FIG. 1. Radioautographs of whole cell lysate (lane 1), acetic acid eluates (lanes 2–4), and SDS eluates (lanes 5–7) analyzed by SDS-PAGE. Affinity columns contained aggregated human IgG (lanes 2 and 5); rabbit IgG anti-OA complexed to OA (lanes 3 and 6); rabbit $F(ab')_2$ anti-OA complexed to OA (lanes 4 and 7). Arrowheads mark the 67,000- and 52,000-dalton proteins unique to eluates from intact IgG columns. In lane 3, heavy chain from IgG anti-OA partially excludes the radioactive 52,000-dalton protein. The additional bands seen in SDS eluates correspond to the most prominent bands found in the original lysate. Dashes to left of lane 1 refer to molecular weight markers 150,000, 80,000, 68,000, and 44,000.

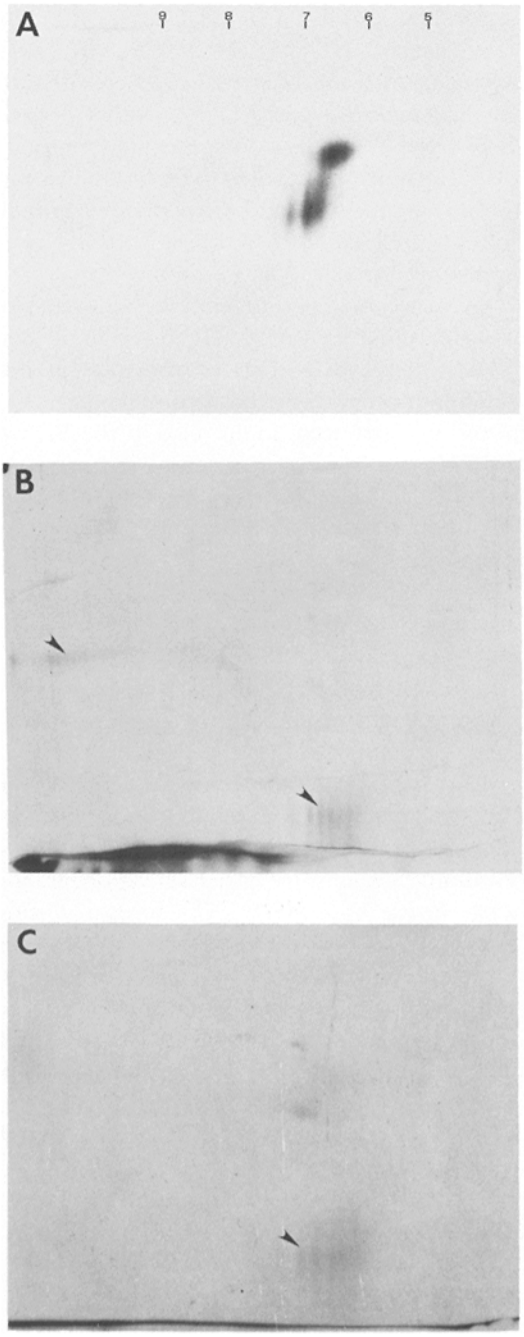

FIG. 2. Two-dimensional gels from acetic acid eluates of IgG (A and B) and F(ab')$_2$ (C) columns. (A) Radioautograph of eluate from rabbit IgG anti-OA complexed to OA. (B) Coomassie blue-stained gel of radioautograph shown in A. (C) Coomassie blue-stained gel of eluate from rabbit F(ab')$_2$ anti-OA complexed to OA. The radioautograph of B showed no radioactive spots. Heavy chain (B) and light chain (B and C) are marked with arrowheads. The pH gradient is shown over A.

6.6; the 52,000-dalton protein also has a broad focusing range between pH 6.6 and 7.5 (Fig. 2A).

IgG Subclass Specificity of the Fc-binding Proteins. To determine if the two Fc-binding proteins represent the IgG subclass-specific Fc receptors present on mouse macrophages, columns of Sepharose 4B coupled to aggregated mouse myeloma proteins of IgG1, IgG2a, IgG2b, and IgG3 were prepared. The lysate was passed over Sepharose 4B-OA charged with $F(ab')_2$ anti-OA and then divided and passed over the IgG subclass myeloma proteins. The columns were sequentially eluted with acetic acid and SDS, and the eluates were analyzed by two-dimensional gels (Fig. 3). The acetic acid eluates from the IgG myeloma columns had very few counts and did not reveal any radioactive spots. SDS eluates from the IgG2a column contained the 67,000-dalton protein (Fig. 3A), whereas SDS eluates from the IgG1 and IgG2b columns contained the 52,000-dalton protein (Fig. 3B). As noted before, SDS eluates contained several additional spots, but these were found also in the SDS eluates from $F(ab')_2$ columns (Fig. 3C). SDS eluates from IgG3 columns did not contain the Fc-binding proteins, but did contain the nonspecifically bound proteins seen in $F(ab')_2$ eluates.

Although the two Fc-binding proteins demonstrate remarkable IgG subclass specificity, a faint spot, which corresponds to the other Fc-binding protein, is occasionally seen in radioautographs of eluates from IgG2a and IgG2b columns when the film exposure time is increased. The eluates from IgG1 columns appear to only contain the 52,000-dalton protein. One explanation of this finding is that the myeloma proteins are contaminated with the other subclass; however analysis of the myeloma proteins by immunodiffusion and IEF did not detect any subclass contamination. An alternative explanation, which is supported by the data on rosette formation of intact cells, is that there is a degree of cross-binding between the IgG2 subclasses for the two receptors, whereas IgG1 may bind exclusively to one receptor. Thus the subclass specificity of the Fc-binding proteins may not be absolute, but does reflect a greater affinity of the two Fc-binding proteins for either IgG2a or IgG2b.

Trypsin Sensitivity of the Fc-binding Proteins. The trypsin sensitivity of the two Fc-binding proteins was assessed by incubating the cells with 1 mg/ml of trypsin for 30 min before lysis. The lysates from treated and untreated cells were sequentially passed over an $F(ab')_2$ column and a column that contained a mixture of Sepharose 4B coupled to IgG2a and IgG1. Two-dimensional gels of the SDS eluates from the myeloma protein column of the untreated cells revealed both Fc-binding proteins (Fig. 4A). The SDS eluates from the trypsin-treated cells lacked the 67,000-dalton Fc-binding protein which has affinity for IgG2a, but contained the trypsin-resistant 52,000-dalton protein which binds to IgG1 and IgG2b (Fig. 4B). In the trypsin-treated cells there appeared to be partial degradation of the 52,000-dalton protein with the appearance of a protein with an ~40,000 mol wt. This is probably a proteolytic product of the 52,000-dalton protein that retains its Fc-binding capability.

Discussion

The existence of separate FcR for IgG2a and IgG2b on mouse macrophages was originally suggested by binding studies with an established line of mouse peritoneal macrophages, IC-21 (6). Using other macrophage-like cell lines, several groups have confirmed and expanded on this finding. Variants of P388D1 (7) and J774 (10) have been isolated that lack one of the receptors and retain the other. In a study that

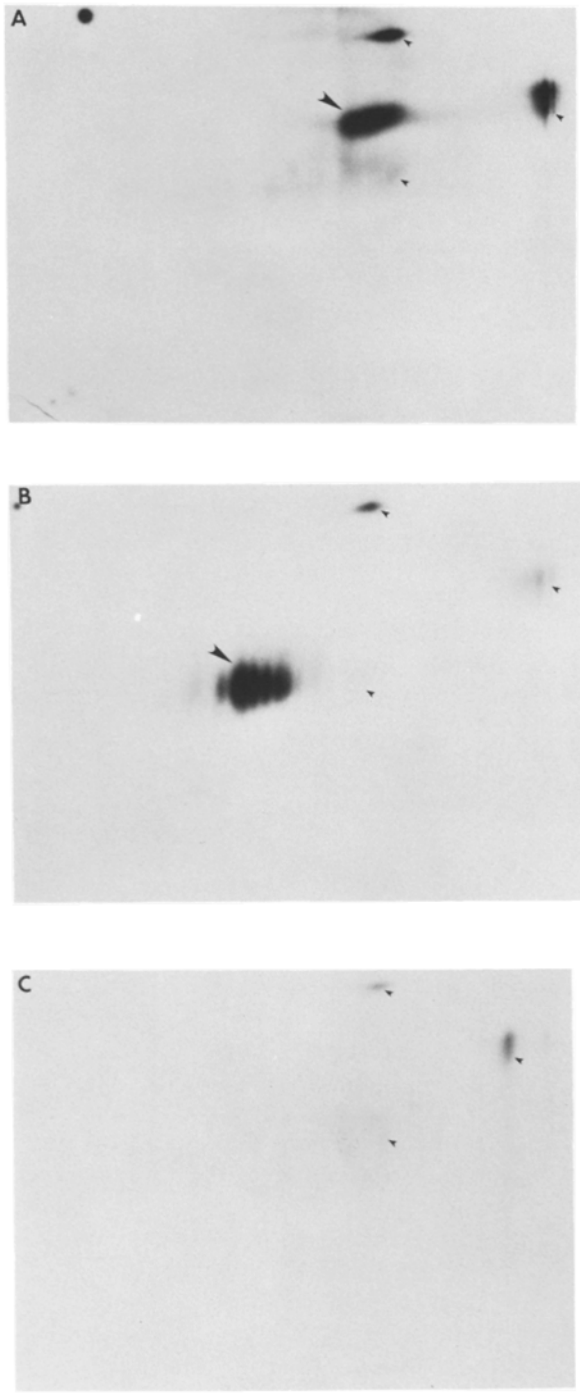

FIG. 3. Radioautographs of two-dimensional gels of SDS eluates from (A) aggregated IgG2a column; (B) aggregated IgG1 column; (C) aggregated $F(ab')_2$ column. Large arrowheads in A and B mark the 67,000- and 52,000-dalton proteins, respectively. Small arrowheads mark the nonspecifically bound proteins that are eluted with SDS.

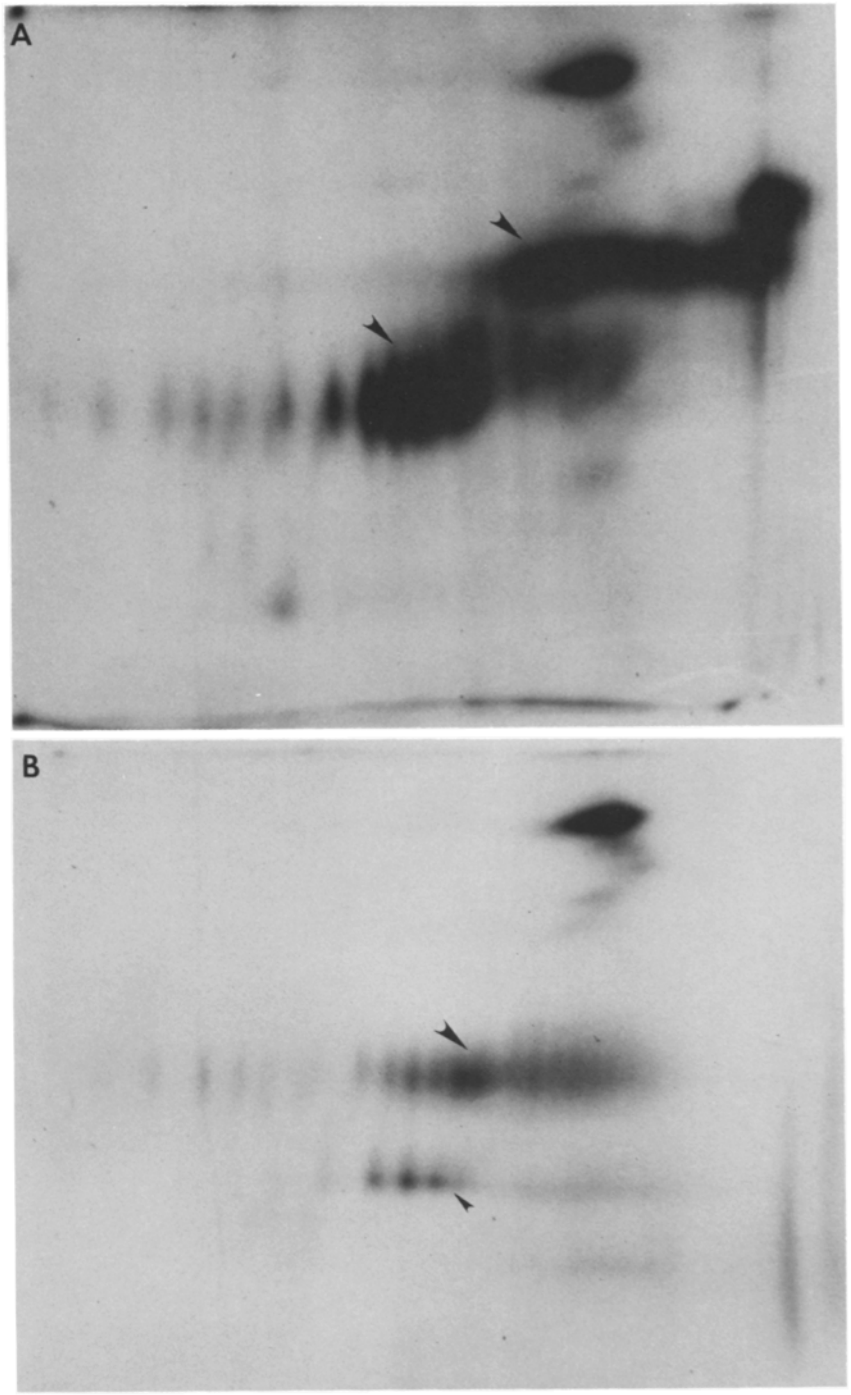

FIG. 4. Radioautograph of two-dimensional gels of SDS eluates from affinity columns containing both IgG2a and IgG1. (A) nontreated cells and (B) trypsin-treated cells. Both Fc-binding proteins (large arrowheads) are present in untreated cells, but the 67,000-dalton protein is absent from trypsin-treated cells. A new spot, probably a degradation product of the 52,000-dalton protein that retains Fc-binding capability, is seen in the trypsin-treated cells (small arrowhead).

primarily dealt with P388, it was found that one FcR bound monomeric IgG2a, and the other bound aggregates of IgG1, IgG2b, and IgG2a (8). In another study of P388D1, it was found that one FcR bound best to monomeric IgG2a, whereas the second interacted better with monomeric IgG2b (11). In this study the receptors showed cross-specificity for monomeric IgG2 subclass proteins, but did not bind monomeric IgG1. A monoclonal antibody has been produced which apparently has specificity for the IgG2b receptor (19).

Studies on peritoneal macrophages are more limited, although the results appear to be similar to those for macrophage-like lines. SRBC sensitized with monoclonal IgG2a and IgG2b anti-SRBC antibodies have been shown to bind to distinct FcR (9). The binding experiments in Table I, demonstrate that the peritoneal macrophages in the present study express the same binding specificity. The vast majority, if not all of the cells, are able to bind IgG2a- and IgG2b-coated erythrocytes. The trypsin effect and selective inhibition with aggregated proteins of the same subclass confirm the existence of a trypsin-sensitive FcR that, preferentially binds antigen-complexed IgG2a and a trypsin-resistant FcR that preferentially binds antigen-complexed IgG2b. These studies further suggest that the two FcR may express some degree of cross-specificity for IgG2a and IgG2b, but that IgG1 appears to be bound only by the trypsin-resistant IgG2b FcR.

Reports of the isolation of IgG FcR from cell types other than macrophages have appeared, but at the present time there is no general agreement on their structure (20–26). Studies limited to the characterization of FcR from macrophages and macrophage-like cell lines have also revealed significant differences (27–33). In the current study we found two membrane proteins from mouse thioglycollate-elicited peritoneal macrophages, with ~52,000- and 67,000-dalton mol wt, that bound specifically to the Fc region of human and rabbit IgG. We do not know if other membrane components, such as lipoproteins or glycoproteins that are not labeled by the Iodogen method, may also be involved with Fc binding or be part of an Fc receptor complex. However, within the limitations of our isolation and analysis system, the two membrane proteins that we identified do appear to bind specifically to the Fc region of IgG. The two proteins were absent from eluates of various $F(ab')_2$ columns, and they were not found in eluates from IgG columns over which we had passed lysates of Fc receptor-negative cells.

There are several lines of evidence that indicate that these Fc-binding proteins represent all or part of the FcR from peritoneal macrophages. These proteins, which were easily distinguished on two-dimensional gels, display selective binding properties for subclasses of mouse IgG myeloma proteins. The 67,000-dalton protein binds to IgG2a, whereas the 52,000-dalton protein binds to IgG1 and IgG2b. Trypsin treatment of the cells before lysis removes the IgG2a-binding protein, although it leaves intact the IgG2b- and IgG1-binding protein. Thus, the soluble Fc-binding proteins contained in the lysates express the same properties as the cell surface FcR. The properties of these molecules are summarized in Table II.

There is some similarity between our results and those reported by others. In a study of P388D1, a major component of 57,000 dalton, and minor components of 28,000 and 24,000 dalton were found to bind to Sepharose coupled to monomeric mouse IgG2a and human IgG1 (27). In vitro labeling of these same cells revealed a more complex pattern of Fc-binding proteins, with a closely spaced doublet of 62,000

TABLE II

Properties of Fc-binding Proteins from Thioglycollate-elicited Mouse Peritoneal Macrophages

	Approximate mol wt	pI range	Effect of treating cells with trypsin	IgG subclass specificity
FcR I*	67,000	6.0–6.6	Binding activity lost	Binds to aggregated IgG2a; does not bind to aggregated IgG1
FcR II	52,000	6.6–7.5	Binding activity retained (possible degradation product also retains binding activity)	Binds to aggregated IgG1 and IgG2b

* Designation FcR I and FcR II is adapted from Unkeless et al. (10).

and 57,000 dalton, and a single band of 12,000 dalton found on reduced gels, and a 110,000-dalton molecule occasionally found on nonreduced gels (32). In another study of P388D1 a broad Fc-specific band of 40,000–60,000 dalton in 9% gels was observed (33). The P388D1-soluble Fc-binding protein in this study appeared to bind to both monomeric IgG2a and IgG2b. There are several possible reasons to account for why we have been able to detect two separate FcR, whereas in the previous reports, one predominant molecular species was detected. The major difference is that the other studies used the macrophage-like cell line P388D1, whereas we used thioglycollate-elicited peritoneal macrophages. Recent experiments in our laboratory have shown that the Fc-binding proteins of P388D1 migrate on SDS-PAGE as a broad band with an ~60,000 mol wt. When analyzed by two-dimensional gels, this single molecular weight band separates into two molecules with distinct isoelectric points (B. C. Lane and S. M. Cooper. Characterization of Fc binding proteins from mouse macrophage-like cell lines. Manuscript in preparation.). Studies are in progress to determine if these molecules with similar molecular weight but different pI express distinct IgG subclass affinity.

The affinity of the soluble Fc-binding proteins for the mouse IgG myelomas appears to be greater than the binding to heterologous IgG, because harsher conditions were required for elution from these columns. The difficulty encountered in eluting the FcR from the myeloma proteins was surprising since it has been reported that the macrophage IgG FcR has a much lower affinity (range: 10^7–10^8 M^{-1} [34]) than the IgE FcR (>6×10^9 M^{-1} [35]), and with the IgE FcR, Kulczycki and Parker (36) have reported successful acid elution and recovery of a molecule with the ability to rebind to IgE. The apparent higher affinity that we encountered may be due to the fact that the myeloma proteins were heat aggregated before coupling them to Sepharose which could result in multivalent attachment. Because of these conditions we have no data about the relative affinity of the soluble FcR for monomer or aggregated IgG, nor can we speculate on the valency of the soluble FcR after lysis of the cells in nonionic detergent.

It is interesting, but not surprising that structurally distinct molecules have evolved to serve as the IgG subclass-specific FcR. A recent study indicates that the IgG2b FcR recognizes a region in the CH2 domain, while suggesting that the IgG2a FcR may recognize a region between the terminal portion of the CH2 domain and the end of the CH3 domain (37). The functional significance of separate IgG subclass-specific FcR remains obscure; however, one study suggests that phagocytosis by IC-21 is achieved primarily through IgM and IgG2a antibodies and, to a lesser extent, by

IgG2b antibodies, whereas extracellular cytolysis is mediated solely by IgG2b antibodies (38). One may speculate that the structural differences demonstrated in the present study may reflect the same type of structural differences that have evolved for heavy chain isotypes. More extensive biochemical analyses of these molecules should provide that information.

Summary

Membrane proteins which selectively bind to the Fc portion of IgG were identified in the Nonidet P-40 extracts of radiolabeled thioglycollate-elicited mouse peritoneal macrophages. Affinity columns of various IgG preparations coupled to Sepharose 4B were used to absorb the Fc-binding proteins. Analysis of the acetic acid or sodium dodecyl sulfate (SDS) eluates from aggregated human IgG or antigen-complexed rabbit IgG columns revealed two Fcγ-specific proteins with apparent 67,000 and 52,000 mol wt. These proteins were not detected in acid or SDS eluates from $F(ab')_2$ columns or in eluates from IgG columns over which were passed lysates of Fc receptor-negative cells.

With the use of affinity columns that contained aggregated mouse myeloma proteins of different IgG subclasses, we found that the 67,000-dalton protein selectively binds to IgG2a, whereas the 52,000-dalton protein binds to IgG1 and IgG2b. Neither protein was found in SDS eluates from IgG3 columns. Trypsin treatment of the macrophages before detergent lysis removed the 67,000-dalton protein, although it leaves intact the 52,000-dalton protein. These results provide structural confirmation for the existence of separate Fc receptors on mouse macrophages and indicate that the two Fc-binding proteins identified in this study represent all or part of the trypsin-sensitive Fc receptor which binds IgG2a and the trypsin-resistant Fc receptor which binds IgG2b and IgG1.

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