CHARACTERIZATION OF A MONOCLONAL ANTIBODY DIRECTED AGAINST MOUSE MACROPHAGE AND LYMPHOCYTE Fc RECEPTORS*

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Fc receptors[1] (FcR) recognize the Fc domain of IgG and are found on macrophages, lymphocytes, and polymorphonuclear leukocytes (for reviews see Kerbel and Davies [1] and Dickler[2]). These receptors enable the effector cells to recognize foreign antigens to which antibodies are bound and may also play a role in immune responsiveness (3). Although there have been reports of isolation of FcR from various cell types, there is as yet no general agreement on structure or properties. Peptides with apparent molecular weights ranging from 125,000 to 28,000 have been isolated by affinity chromatography on antibody-coated columns (4–9). Indeed, it has been suggested that FcR may not be a protein at all, but represent only hydrophobic interaction of immunoglobulin Fc domains with membrane phospholipid (10).

The situation is further complicated by the presence of 2 Fc receptors on mouse macrophages and macrophage-like cell lines, distinguished by their protease sensitivity and specificity for different mouse IgG subclasses. FcRI, which is inactivated by trypsin, binds monomeric and immune aggregates of IgG2a, whereas FcRII binds immune aggregates of mouse IgG1 and IgG2b and is unaffected by trypsin or pronase (11–15).

Monoclonal antibodies with defined specificity (16) offer a new method with which to analyze the structure and function of well-defined antigens. To examine the nature and specificity of Fc receptors, I isolated and characterized a monoclonal rat anti-FcR antibody. This antibody inhibits the rosetting of antibody-coated erythrocytes to macrophages, macrophage-like cell lines, and lymphocytes, and binds to a macrophage cell line, J774, but not to a set of variants which lack FcRII (17).

Materials and Methods

Cell Culture. The P388D$_1$ macrophage line, P388 lymphoma, J774 macrophage cell line, and FcRII-deficient variants of J774 were grown as described previously (11, 12, 17). E1–4 Bu was provided by the Cell Distribution Center (Salk Institute, San Diego, Calif.); TLX-9, PU5.1,

[1] *Abbreviations used in this paper:* αMEM, alpha-modified minimum Eagle's medium; DNP, dinitrophenyl; DMEM, Dulbecco's modified Eagle's medium with 4.5 g/liter of glucose; E, sheep erythrocytes; E$_{ox}$, ox erythrocytes; E$_{TNP}$, TNP-derivatized sheep erythrocytes; FcR, Fc receptor; HIFBS, heat-inactivated fetal bovine serum; HIHS, heat-inactivated horse serum; PBS, phosphate-buffered saline; PBS-BSA, PBS containing 1 mg/ml bovine serum albumin; PMN, polymorphonuclear leukocyte; TNP, trinitrophenyl; TRITC, tetramethyl rhodamine isothiocyanate.

and P388 were obtained from Dr. Carl Nathan (The Rockefeller University, New York); and WEHI-231 and L5178Y were a gift of Dr. Peter Ralph (Sloan-Kettering Institute, Rye, New York). All cell lines were grown in alpha-modified minimum Eagle's medium (αMEM, K. C. Biological Inc., Lenexa, Kans.) supplemented with 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.) heat inactivated at 56°C for 30 min (heat-inactivated bovine serum [HIFBS]), 100 U/ml penicillin, and 100 μg/ml streptomycin. PU5.1 and WEHI-231 required for growth, in addition, 5 × 10^{-5} M 2-mercaptoethanol. P3U1, a derivative of P3-x6-Ag8 (16) which does not make heavy chains, was kindly provided by Dr. Matthew Scharff (Albert Einstein Medical School, New York) and maintained in Dulbecco's modified Eagle's medium with 4.5 gm/liter of glucose (DMEM) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% HIFBS and 10% heat-inactivated horse serum (HIHS) (Flow Laboratories, Inc.), penicillin, and streptomycin.

Peritoneal cells from unstimulated 20–35 gm ♀ mice were obtained by lavage as described (18). Mouse spleen cells were prepared by gently teasing the spleen to obtain a single cell suspension. Erythrocytes were lysed by incubation for 10 min in cold 0.17 M NH$_4$Cl. Rat, rabbit, and guinea pig peritoneal cells were obtained by lavage 4 d after intraperitoneal injection of 10 ml (for the rat) or 25 ml of 1% proteose peptone broth. Human mononuclear cells were obtained from peripheral blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) as described by Boyum (19). Human granulocytes were isolated as described by English and Anderson (20).

Preparation of Opsonized Erythrocytes. Sheep erythrocytes were incubated with the monoclonal anti-E IgG2a UN-2M2 (EIgG2a) or the monoclonal anti-E IgG2b U88 (EIgG2b) (gifts of Dr. Betty Diamond, Albert Einstein Medical School, Bronx, N. Y.) at nonhemagglutinating titer (21). Ox erythrocytes (E$_{ox}$) were opsonized with a purified rabbit anti-E$_{ox}$ IgG as previously described (22). E derivatized with trinitrophenyl (TNP) groups were prepared as described by Rittenberg and Pratt (23), and were opsonized (21) with a nonhemagglutinating concentration of CD2F$_1$ mouse (BALB/c × DBA/2) anti-dinitrophenyl (DNP) IgG1.

Cell Fusion to Produce Monoclonal Antibodies. A female Sprague Dawley rat was primed by intraperitoneal injection on 6 × 10^7 viable J774 cells, boosted 2 wk later by intraperitoneal injection of a mixture of 3 × 10^7 J774 and 3 × 10^7 P388D$_1$ cells, and sacrificed 3 d later. The spleen was teased and erythrocytes in the single cell suspension were lysed in cold 0.17 M NH$_4$Cl for 10 min. Washed spleen cells were fused with washed P3U1 mouse myeloma cells at a 10:1 spleen:myeloma cell ratio using 30% polyethylene glycol 1000 (J. T. Baker Chemical Co., Phillipsburg, N. J.) dissolved in DMEM according to Gefter et al. (24). The products of the fusion were dispensed into 100 μl portions in flat-bottom wells (Costar 3596, Costar Data Packaging, Cambridge, Mass.) at a density of 1 × 10^6 P3U1 cells/ml in DMEM with 10% HIFBS and 10% HIHS containing hypoxanthine, aminopterin, and thymidine (100, 10, and 30 μM, respectively). The cells were fed every 5 or 6 d with 50 μl of medium containing hypoxanthine, aminopterin, and thymidine and screened after 18 d for production of anti-Fc receptor antibody.

J774 cells (3 × 10^3 cells in 5 μl) were seeded in Terasaki trays (Falcon 3034, Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) and incubated for 30 min on ice with 5 μl hybridoma supernate, rinsed in phosphate-buffered saline (PBS; NaCl, 0.137 M; KCl 3 mM; Na$_2$HPO$_4$, 16 mM; and KH$_2$PO$_4$, 2 mM) and 5 μl of 1% (vol/vol) EIgG2b in PBS were added. After 20 min at 37°C to allow rosettes to form, nonadherent EIgG2b were removed by inverting the trays in PBS for 10 min and the wells were then examined for inhibition of rosetting. Cells in wells of interest were cloned in 0.5% sea plaque agarose (Marine Colloids, Inc., Rockland, Me.) over a monolayer of rat fibroblasts (Microbiological Associates, Walkersville, Md.) seeded initially at 5 × 10^4 cells/10-cm diameter tissue culture dish (Falcon 3003, Falcon Labware, Div. of Becton, Dickinson, & Co.). Clones were picked by micropipette, placed in cloning wells, and rescreened after adequate growth.

To make large amounts of monoclonal antibody, 3 × 10^6 hybridoma cells were injected intraperitoneally into CD2F$_1$ mice (Flow Laboratories, Inc.) which had been injected intraperitoneally 1–3 wk previously with 0.5 ml of 2, 6, 10, 16-tetramethyl-pentadecane (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The mice were irradiated with 500 rad (^{131}Cs, 128 rad/ min) immediately before injection with the hybridoma cells. The ascites fluid was tapped every 2 d and frozen.

Preparation of Immunoglobulins and Proteolytic Fragments.   Ascites fluid from the 2.4G2 hybridoma was adjusted to 45% saturation with $(NH_4)_2SO_4$. The precipitate collected by centrifugation was dissolved in PBS and subjected to Sephadex G-200 chromatography (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.). The IgG peak from the Sephadex G-200 column was dialyzed against 0.025 M Tris-HCl, pH 8 and 100 mg of protein applied to a 2 × 5-cm column of DE-52 (Whatman, Inc., Clifton, N. J.). The column was eluted with a 500 ml 0–0.5 M gradient of NaCl dissolved in 0.025 M Tris-HCl, pH 8. The 2.4G2 IgG eluted at 0.1 M NaCl. To prepare the Fab fragment, the purified IgG was digested with a 1:100 wt/wt ratio of papain for 16 h at 37°C as described by Porter (25). The digest was purified on a DE-52 column as described above. The Fab fragment eluted at 0.09 M NaCl.

The mouse IgG2a myeloma protein LPC-1 and rabbit anti-DNP IgG were prepared as described previously (11, 12).

CD_2F_1 mouse anti-DNP IgG was prepared from ascites fluid from mice immunized repeatedly with DNP-keyhole limpet hemocyanin as described by Tung et al. (26). The anti-DNP IgG from the ascites fluid was isolated as described (12). To isolate IgG_1, which does not bind to protein A (27), anti-DNP IgG (4.5 mg) was passed sequentially over two 0.3-ml columns of protein-A Sepharose 4-B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.). Of the purified anti-DNP IgG initially loaded on the column, 0.54 mg of anti-DNP IgG1 was isolated. The anti-DNP IgG1 formed precipitin lines in agar with only goat anti-mouse IgG1 (IgG1 antiserum, lot 88336; IgG2a antiserum, lot 88570; IgG2b antiserum, lot 77028, Meloy Laboratories, Inc., Springfield, Va.; IgG3 antiserum, lot 231-70-9, Litton Bionetics, Kensington, Md.).

Iodinations.   Proteins (50 μg each) were iodinated with 500 μCi of ^{125}INa (Amersham Corp., Arlington Heights, Ill.) using chloramine T at a molar chloramine T:IgG ratio of 10:1 as described (11, 28). Incorporation of ^{125}I into protein was >50%.

Immunofluorescence.   Viable adherent cells were seeded on glass coverslips. Nonadherent cells in medium without serum were centrifuged (200 g, 10 min) onto 12-mm diameter acid-washed coverslips which had previously been incubated for 30 min in 50 μg/ml poly-L-lysine (34,000 mol wt, Sigma Chemical Co., St. Louis, Mo.), dissolved in PBS, and then rinsed in water. After the cells had been centrifuged onto the slips, they were transferred to medium with serum. The coverslips were then incubated with 2.4G2 IgG (30 μg/ml) or a 1:200 dilution of ascites in PBS containing 1 mg/ml of crystallized bovine albumin (PBS-BSA) for 30 min at 4°C. After gentle rinsing, the cells were stained for 30 min at 4°C with a 1:20 dilution of a tetramethyl rhodamine isothiocyanate (TRITC)-rabbit anti-rat IgG F(ab')₂ reagent (N. L. Cappell Laboratories, Inc., Cochranville, Pa.), rinsed, and fixed in 10% buffered neutral formalin. Cells were screened for surface IgM by staining with a fluorescein isothiocyanate-coupled rabbit anti-μ reagent (29). Specimens were viewed with a × 63 oil immersion lens on a Zeiss microscope with epifluorescence optics (Carl Zeiss, Inc., N. Y.).

Immunoelectrophoresis and Acrylamide Gel Electrophoresis.   Immunoelectrophoresis was performed in 2% agar, 0.05 M Na barbital, pH 8.2, and the slide developed with a rabbit anti-rat IgG (N. L. Cappell Laboratories, Inc.). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed as described by Neville (30) using 1-mm thick slab gels with a 4–11% gradient of acrylamide. Sample buffer was 50 mM Na_2CO_3 pH 8.4, 2% SDS, 50 mM dithiothreitol, 12% wt/vol sucrose.

Binding Studies.   Washed cells (5–10 × 10⁵) were incubated in suspension with ^{125}I-2.4G2 IgG in PBS-BSA at 4°C for 1 h. After dilution with 3 ml cold PBS-BSA, they were centrifuged at 500 g for 5 min, and the supernate was removed. The pellet was washed with 3 ml of PBS-BSA, recentrifuged, and the tubes assayed for radioactivity after removal of the supernate. The washing removed 99.4% of input radioactivity from both empty tubes and negative cell lines. Experiments were usually carried out using a constant trace amount of ^{125}I-2.4G2 IgG with variable amounts of nonradioactive 2.4G2 IgG so that the specific activity was between 10⁴ and 10⁶ cpm/μg.

Results

Characterization of the Monoclonal 2.4G2 IgG.   The screen which was used to search for an anti-FcR monoclonal antibody was inhibition of the binding of EIgG2b to J774 cells seeded in Terasaki plates. Of five separate fusions with immunized rat

PROTEIN AUTORADIOGRAM
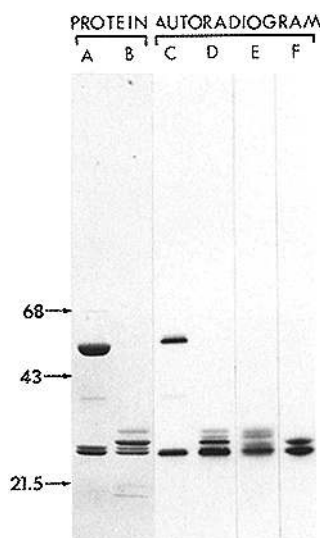
A B C D E F

68—►

43—►

21.5—►

FIG. 1. Characterization of the purified 2.4G2 IgG molecule and Fab fragments. Samples were reduced with 50 mm dithiothreitol and boiled for 1 min before SDS polyacrylamide gel electrophoresis. Lanes A and B, Coomassie Brilliant Blue stain; lanes C–F, autoradiogram of ^{125}I-proteins. Lane A, intact 2.4G2 IgG; lane B, Fab fragment; lane C, ^{125}I-2.4G2 IgG; lane D, ^{125}I-Fab; lane E, final supernate from J774-adsorbed ^{125}I-Fab (Table I); lane F, ^{125}I-Fab adsorbed to J774 cells in first cycle (Table I). Autoradiograms were exposed on Dupont Cronex x-ray film for 1 d. Standards used to determine molecular weights were soybean trypsin inhibitor (21,500 daltons), ovalbumin (43,000 daltons), and BSA (68,000 daltons).

spleen cells, which yielded >1,000 hybridoma clones, only one hybridoma-making antibody of the desired specificity was isolated, 2.4G2. Of the >100 clones with secreted anti-macrophage antibodies, determined by immunofluorescence, none had any effect on EIgG2b rosette formation.

The purified 2.4G2 hybridoma antibody had one type of heavy chain, of apparent mol wt 52,000, and two different types of light chains, one of which derived from the P3U1 myeloma, and one which came from the rat spleen B cell (Fig. 1 A). After digestion with papain there was no visible intact heavy chain, and two heavy chain fragments, of apparent mol wt 30,000 and 26,000 were seen (Fig. 1 B). Judged by Coomassie Blue staining, the relative abundance of these two new fragments was approximately the same as the relative abundance of the two light chains, which were not altered in electrophoretic mobility after papain digestion. This result suggests that the type of light chain associated with the heavy chain determined the extent of papain digestion of the latter.

To ascertain which type of light chain in the hybridoma IgG was of rat spleen cell origin, the purified Fab fragments were iodinated, and sequential adsorptions were carried out with either J774 cells or a non-FcR-bearing lymphoid line, L5178Y. The results (Table 1) show that >50% of the ^{125}I incorporated into the Fab fragments was bound to J774 cells. The amount bound in the third adsorption was only 20% of that bound in the first. Little radioactivity (0.5% of the initial ^{125}I) was adsorbed by L5178Y cells. The ^{125}I-Fab adsorbed to the first aliquot of J774 cells and the supernate from the final adsorption were reduced and analyzed by SDS polyacrylamide gel electrophoresis. The autoradiogram (Fig. 1 E) shows clearly that the more rapidly

TABLE I

Sequential Adsorption of ^{125}I-2.4G2 Fab Fragments by J774 and L5178Y Cells

Treatment	J774 macrophage cell line	Lymphoma
	cpm × 10^{-5}	
Initial	4.51	4.66
Adsorption 1	1.39	0.014
Adsorption 2	0.81	0.005
Adsorption 3	0.25	0.004
Total washes	0.50	0.64
Final supernate	1.57	4.00

Aliquots of ^{125}I-2.4G2 Fab (5 µl, 20 µg/ml, 5 × 10^3 cpm/ng) diluted with 20 µl of PBS-BSA were sequentially adsorbed three times with fresh portions of J774 or L5178Y cells (2 × 10^5 cells per adsorption). The cell pellet after each cycle was resuspended and washed with 1 ml of cold PBS-BSA. Adsorption 1 of J774 cells and the final J774 supernate were subjected to polyacrylamide gel electrophoresis and are shown in Fig. 1, F and E, respectively.

migrating H-chain fragment was adsorbed from the ^{125}I-Fab (Fig. 1 D). Only the Fab fragments composed of the rapidly migrating heavy chain fragment—light chain pair bound to the J774 cells (Fig. 1 F). Assuming that the homologous rat light chain-rat heavy chain combination results in the formation of the antibody binding site, this experiment defined the rat light chain as the more rapidly migrating peptide.

The intact hybridoma antibody was tested by Ouchterlony diffusion against goat anti-IgM, IgE, IgA, and IgG antisera, generously supplied by Doctors Teruko and Kimishige Ishizaka (Johns Hopkins School of Medicine, Baltimore, Md.), and formed a precipitin line only with anti-IgG (not shown). The 2.4G2 IgG does not bind to protein A (not shown). Of the four rat IgG subclasses, Medgyesi et al. (31) have shown that only rat IgG2a and IgG2b fail to bind to protein A. However, the assignment of the hybridoma IgG to either of these subclasses must be provisional, because both are reported to migrate rapidly towards the cathode (32), and 2.4G2 IgG migrates very slowly relative to the bulk of rat IgG (Fig. 2).

Inhibition of Macrophage FcR. Preincubation of J774 cells with different dilutions of 2.4G2 ascites fluid followed by rinsing to remove unbound hybridoma antibody resulted in striking inhibition of EIgG2b binding to Fc receptors (Figs. 3 b, c). This inhibition was observed even at a 1:16,000 dilution of the original ascites (Fig. 3 d). To test the possibility that the inhibition of the Fc receptors was not a result of the hybridoma binding to the cell surface via the antibody combining site but a result of the blockade of Fc receptors by the Fc domain of the hybridoma antibody, the Fab fragment of 2.4G2 IgG was prepared.

The Fab fragment of 2.4G2 IgG inhibited the binding of EIgG2b to macrophages (Fig. 4 b) relative to the control (Fig. 4 a), although not to the same extent as the intact hybridoma antibody (Fig. 4 c). The monovalent Fab fragment probably has a lower affinity for the antigenic site than the bivalent 2.4G2 IgG, and thus does not inhibit EIgG2b rosette formation as efficiently. Similar results were obtained using E derivatized with TNP groups which were then coated with mouse anti-DNP IgG1 (ETNP IgG1). These erythrocytes bound avidly to J774 cells (Fig. 4 d) but did not bind to J774 cells preincubated with either the Fab fragment (Fig. 4 e) or the intact hybridoma antibody (Fig. 4 f). Thus, the inhibition of rosetting of EIgG2b and ETNP IgG1 to macrophages by the monoclonal antibody was a result of the antigenic
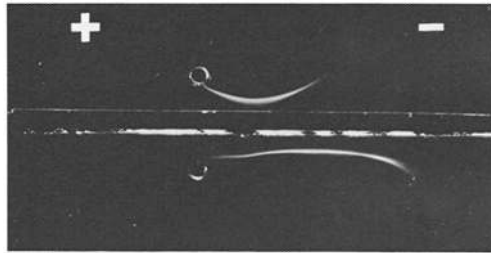
Fig. 2. Immunoelectrophoresis of 2.4G2 IgG and rat serum. The top well was loaded with 15 μl of 1.15 mg/ml 2.4G2 IgG, the bottom well with 15 μl normal rat serum. After electrophoresis, the center well was filled with rabbit anti-rat IgG, and developed overnight.

binding site of the antibody, and not to immune blockade of Fc receptors by the Fc domain.

A different pattern of Fc receptor inhibition by the hybridoma antibody and Fab fragment was observed when EIgG2a binding to macrophages was tested. Rosette formation with EIgG2a was blocked by preincubation with 2.4G2 IgG (Fig. 4j) but was unaffected by preincubation with the Fab fragment (Figs. 4 g, h). We also tested the binding of monomeric ^{125}I-IgG2a (11) to J774 cells after preincubation of the J774 cells with 2.4G2 IgG or the Fab fragment. There was no inhibition by the F(ab) fragment, and the intact hybridoma antibody inhibited binding of monomeric IgG2a totally (not shown). Thus, the inhibition of IgG2a and EIgG2a binding by the hybridoma antibody was a result of Fc domain blockade of the FcRI site. The antigenic site to which 2.4G2 IgG binds is unrelated to the IgG2a-specific Fc receptor site. It appears likely that mouse IgG2a, whether aggregated or monomeric, binds to a receptor distinct from that which binds aggregated IgG2b, as discussed by Diamond et al. (14).

Binding of 2.4G2 IgG to Macrophage FcR Variants. A set of variants of the J774 mouse macrophage cell line had been previously isolated which bound only 1-3 E coated with IgG2b compared to 25 or more for the J774 parent. The variants lacked the trypsin-resistant FcR for IgG2b aggregates (FcRII), but not FcRI which binds mouse IgG2a (17). The binding of the 2.4G2 IgG to these variants and the parent cell line was examined by immunofluorescence using a TRITC-rabbit anti-rat IgG F(ab')₂ reagent to stain viable cells first incubated with 2.4G2 IgG (Fig. 5). The variants showed a dramatic lack of immunofluorescence compared to the parent, indicating the absence of the antigenic determinant recognized by 2.4G2 IgG.

These experiments were repeated using a quantitative assay of the binding of ^{125}I-2.4G2 IgG to the same cell lines. A preliminary experiment established that there was no detectable dissociation of bound ^{125}I-2.4G2 IgG from the surface of J774 cells over a 3-h period (not shown). The results of the quantitative binding experiment (Fig. 6) confirmed the immunofluorescence data. The parent J774 cells bound 0.15 μg of 2.4G2 IgG per 10^6 cells, ~600,000 IgG molecules per macrophage. The binding to J774 cells of ^{125}I-Fab fragment was similar to the intact 2.4G2 IgG except that slightly less of the Fab fragment bound at saturation. Trypsinization of the J774 cells had no effect on subsequent binding of ^{125}I-2.4G2 IgG.

Because the variants were all isolated from independent mutagenizations of J774
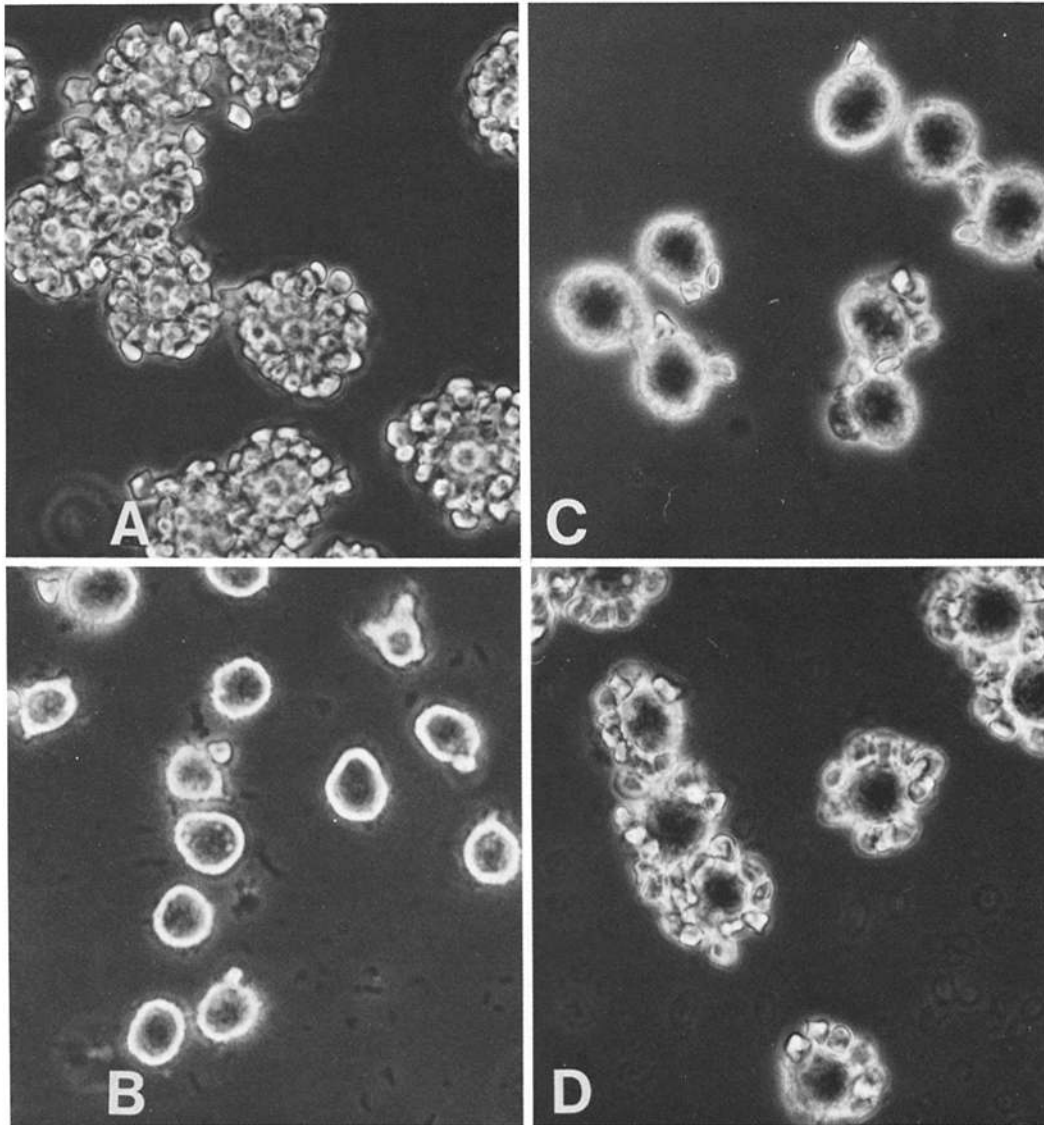
Fig. 3. Inhibition of rosetting with EIgG2b by preincubation of J774 cells with 2.4G2 ascites fluid. A, control; B, 1:1,000 ascites dilution; C, 1:4,000 ascites dilution; D, 1:16,000 ascites dilution. J774 cells on coverslips were preincubated for 1 h on ice with ascites dilutions, rinsed in PBS, and incubated with 1% EIgG2b at 37°C for 20 min.

cells and were all markedly defective in 2.4G2 IgG binding, these results strengthened the probability that the 2.4G2 IgG was directed against the FcRII receptor or a closely associated structure. Assuming that each 2.4G2 IgG molecule can inhibit two binding sites, the number of FcRII sites on J774 cells was ~1.2 $\times$ 10^6 per cell, four times greater than the number of IgG2a FcRI sites (3.1 $\times$ 10^5) on J774 (17).

Binding of 2.4G2 IgG and Inhibition of FcR on Macrophages and Other Cells. An advantage of monoclonal sera over conventional antisera is that they can be used to
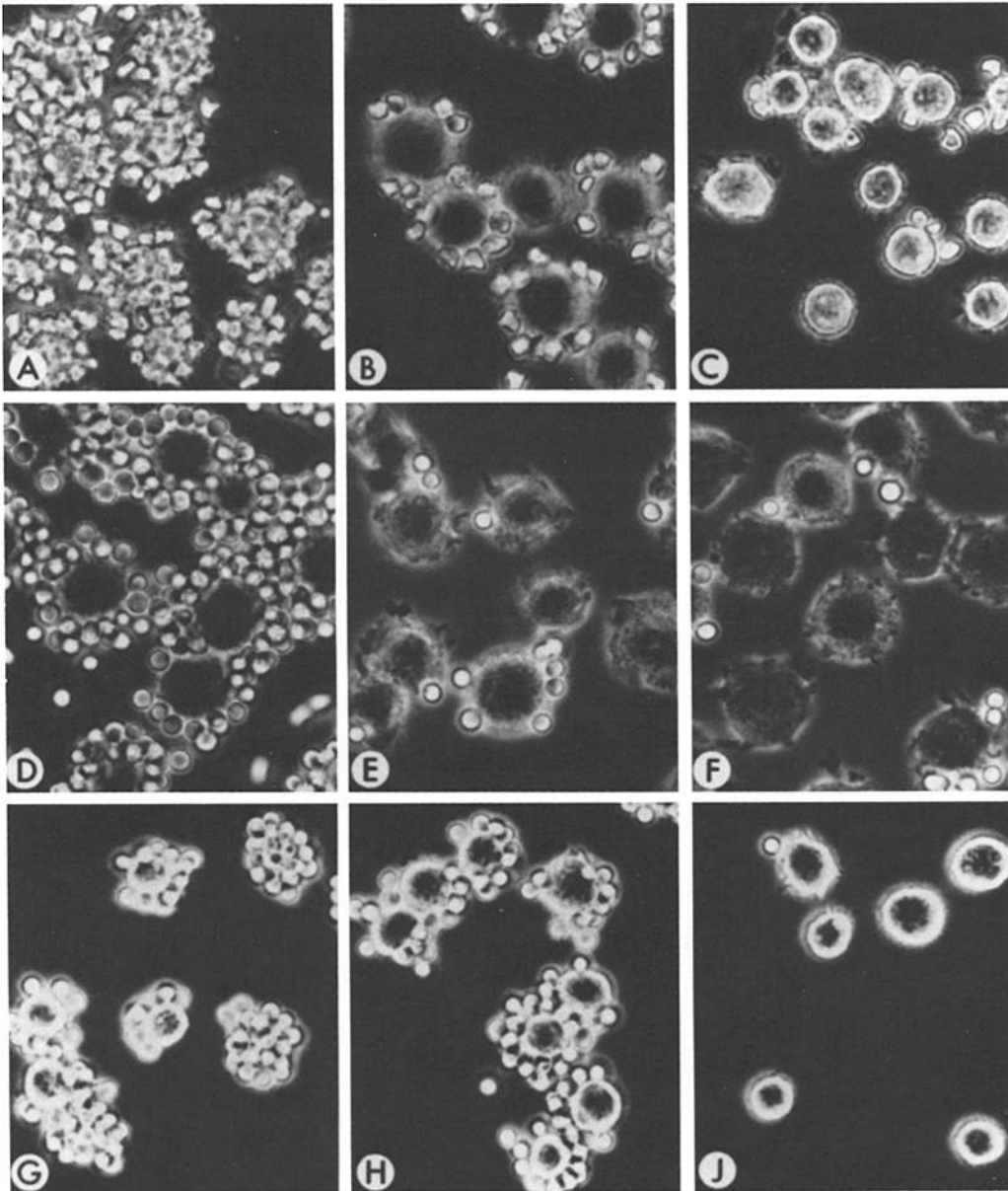
Fig. 4. Binding of E coated with different subclasses of mouse IgG to J774 cells preincubated with 2.4G2 IgG or Fab. A–C, rosetting of EIgG2b on A, J774 cells; B, J774 cells preincubated with 2.4G2 Fab; C, J774 cells preincubated with 2.4G2 IgG. D–F, rosetting of $E_{TNP}IgG1$ on D, J774 cells; E, J774 cells preincubated with 2.4G2 Fab; F, J774 cells preincubated with 2.4G2 IgG. G–J, rosetting of EIgG2a on G, J774 cells; H, J774 cells preincubated with 2.4G2 Fab; J, J774 cells preincubated with 2.4G2 IgG. Preincubations were for 30 min at 4°C with 30 μg/ml of IgG or Fab in PBS-BSA.

define a unique antigenic determinant on a variety of cell types without the necessity of adsorption to remove unwanted specificities. First a series of cell populations was screened for binding of 2.4G2 IgG by immunofluorescence. Binding was detected using a TRITC-rabbit anti-rat IgG $F(ab')_2$ as the second reagent. In this assay
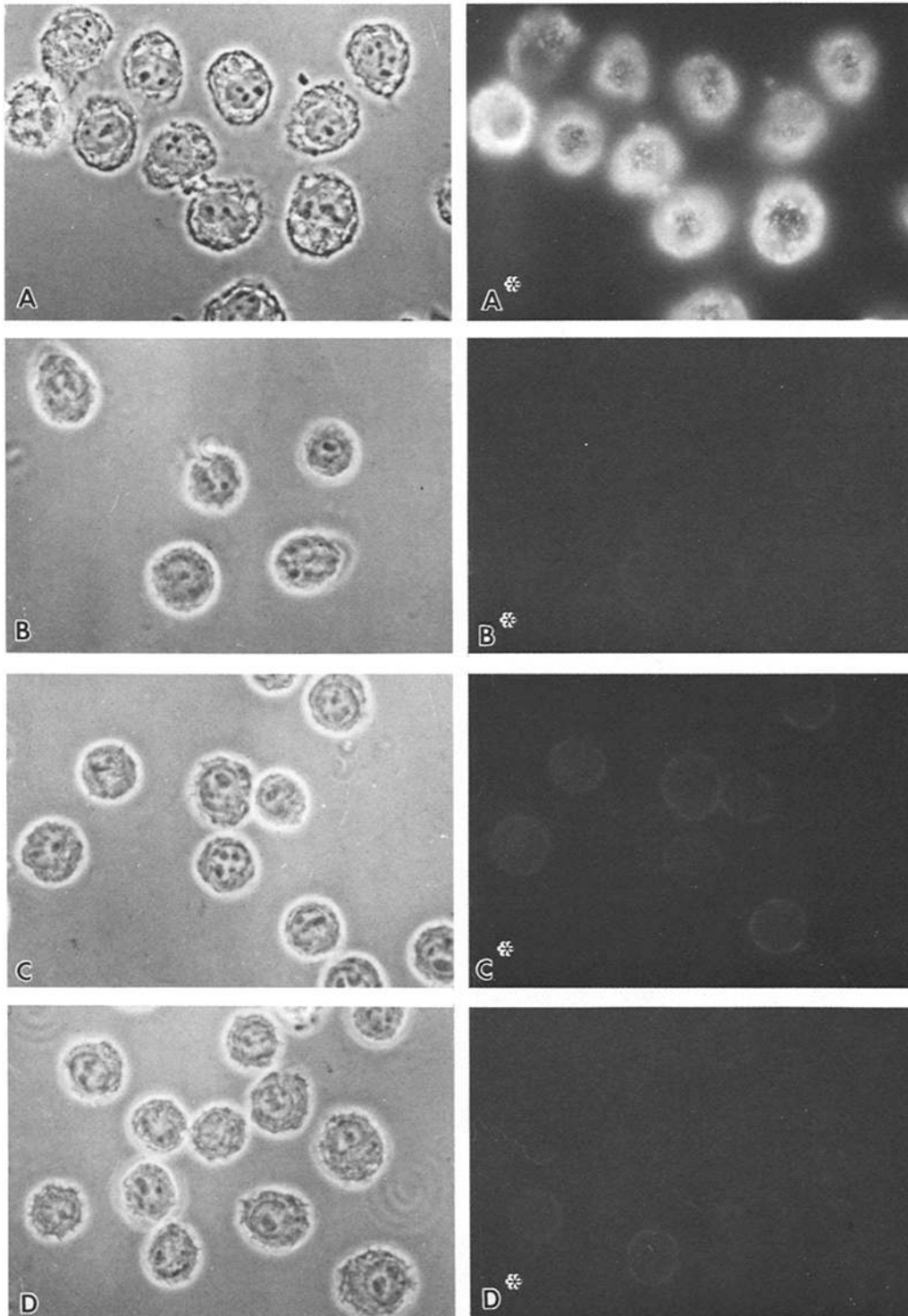
Fig. 5. Binding of 2.4G2 IgG to J774 cells and FcRII variants detected by immunofluorescence. Photographs on the left were taken with phase-contrast optics, photographs on the right, with epifluorescence optics. A, J774 cells; B, ICR7.1 cells; C, MNNG1.2 cells; D, MNNG 6.31 cells. Fluorescence photographs were all 3-min exposures.
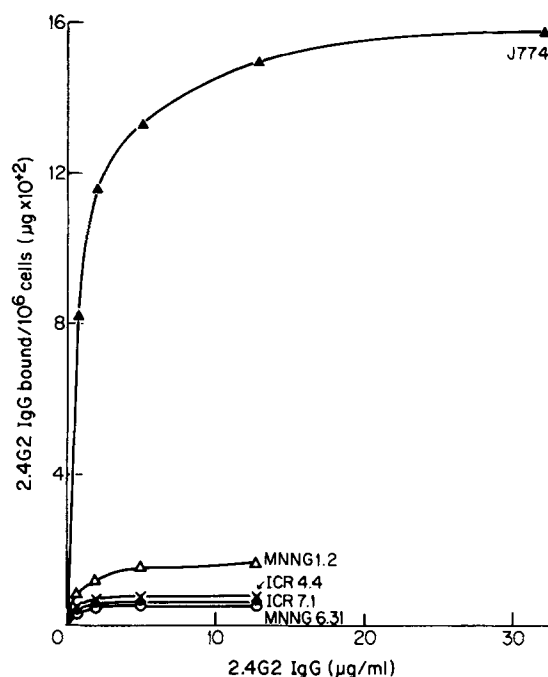
Fig. 6. Quantitative analysis of binding of ^{125}I-2.4G2 IgG to J774 cells and different FcRII variants. Cells (10^6) were incubated for 1 h at $4°C$ with 0.25 ml of PBS-BSA containing a constant amount of ^{125}I-2.4G2 IgG (5×10^3 CPM, 5.6 $\times$ 10^3 CPM/ng) and nonradioactive 2.4G2 IgG as noted. The cells were then washed and assayed for bound radioactivity. Radioactivity bound to serum-coated empty tubes was subtracted.

macrophages, ~40% of spleen cells, the J774 and P388D₁ macrophage-like cell lines, and the P388 lymphoma bound 2.4G2 IgG (not shown). Negative cells included thymocytes, L-cells, and the P3U1 myeloma. To obtain more quantitative data, the binding of ^{125}I-2.4G2 to a series of mouse lymphoid lines was studied (Fig. 7). Cells binding 2.4G2 IgG were the P388 lymphoma, PU5.1, and WEHI-231. Cells which did not bind 2.4G2 IgG (giving a background of binding identical to serum-coated empty tubes) were L5178Y, EL-4, TLX-9, thymocytes, and splenic dendritic cells isolated by the procedure of Steinman et al. (33).

In addition to screening for the binding of 2.4G2 IgG, the ability of the monoclonal antibody to inhibit rosetting was also examined. These experiments were done using opsonized ox erythrocytes (E$_{ox}$IgG), which can be coated with a higher density of IgG than sheep erythrocytes without agglutinating (34) and thus are a more sensitive indicator than EIgG for FcR. Table II shows that the cells which bound 2.4G2 IgG formed rosettes (J774, P388, PU5.1, and WEHI-231) and that preincubation with the monoclonal antibody blocked the binding of E$_{ox}$IgG. The extent of rosetting was roughly proportioned to the amount of 2.4G2 IgG bound. Rosette formation by WEHI-231, which bound only 30,000 molecules of the hybridoma IgG per cell, could not be demonstrated using EIgG, and was weak even with E$_{ox}$IgG. The extent of rosetting by EL-4, L5178Y, TLX-9, and thymocytes was very low and, with the exception of thymocytes, where ~1% of the cells formed rosettes with >11 E, no heavily rosetted cells were found.

To rule out the possibility that antigen recognized by 2.4G2 IgG was present only
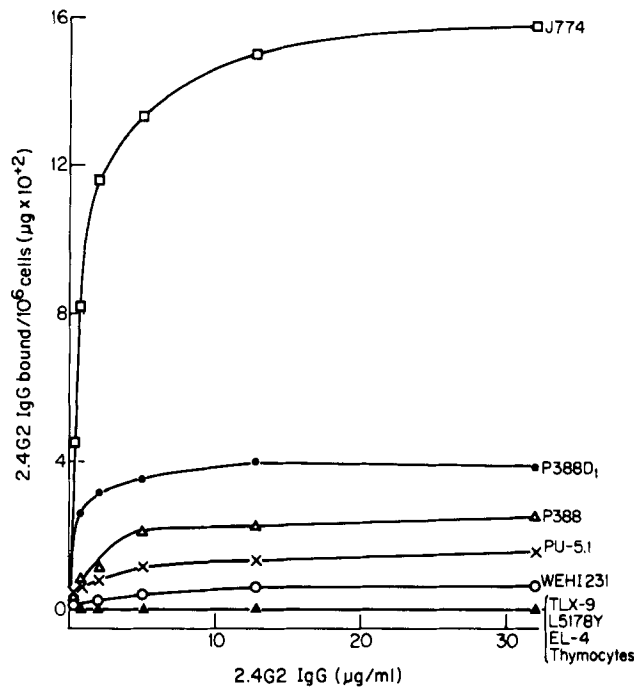
y-axis: $2.4G2$ IgG bound/10^6 cells (μg $\times 10^{+2}$)

x-axis: $2.4G2$ IgG (μg/ml)

Curves labeled: J774, P388D$_1$, P388, PU-5.1, WEHI231, TLX-9, L5178Y, EL-4, Thymocytes

FIG. 7. Quantitative analysis of binding of ^{125}I-2.4G2 IgG to different cell lines. Procedures were as detailed in Fig. 6.

TABLE II

Inhibition of E_{ox}IgG Rosette Formation on Lymphoid and Macrophage Cell Lines by 2.4G2 IgG

Cell	Phag-ocyto-sis of latex	Lyso-zyme secre-tion	Sur-face Ig	Thy-1	Classification	Preincuba-tion	Ro-settes	Bound E_{ox}IgG
							% of total	per cell
J774 (12, 46, 48)	+	+	−	−	Macrophage	−	85	9.8
						2.4G2 IgG	15	1.0
P388 (47, 48)	−	−	−	−	Null lymphoid	−	73	5.5
						2.4G2 IgG	12	0.4
PU5.1 (48, 49)	−	−	−	−	(B cell)*	−	79	6.3
						2.4G2 IgG	8	0.3
WEHI-231 (50)	−	−	+	−	B cell	−	53	3.0
						2.4G2 IgG	19	0.8
EL-4 (13, 47, 48)	−	−	−	+	T cell	−	16	0.6
						2.4G2 IgG	10	0.4
L5178Y (13, 51)	−	−	−	+	T cell	−	11	0.4
						2.4G2 IgG	10	0.4
TLX-9 (51)	−	−	−	?	Lymphoid	−	3	0.1
						2.4G2 IgG	4	0.2
Thymocytes	−	−	−	+	T cell	−	11	0.6
						2.4G2 IgG	3	0.1

Cells were preincubated in PBS-BSA containing 30 μg/ml of 2.4G2 IgG or Fab for 30 min on ice.
After washing by centrifugation, a 40-fold excess of E_{ox}IgG was added and the mixture of cells was centrifuged to form a pellet in round-bottom plastic tubes. After a 1-h incubation on ice, the cells were gently resuspended by pipette and counted. Cells binding three E_{ox}IgG or more were counted as rosettes.
* Although PU5.1 has been reported to have surface μ-chain, none was detected on the cells I tested.

TABLE III

Inhibition of E₀ₓIgG Binding in Spleen and Peritoneal Cell Populations by 2.4G2 IgG and the 2.4G2 Fab Fragment

Strain of mouse	H-2 haplo-type	Cell population	Preincubation	Rosettes	E₀ₓIgG per cell
				% of total	
C57/B16	b	Peritoneal	Control	27	2.0
		Peritoneal	2.4G2 IgG	4	0.2
		Spleen	Control	47	3.2
		Spleen	2.4G2 IgG	5	0.3
DBA/2J	d	Peritoneal	Control	21	1.4
		Peritoneal	2.4G2 IgG	4	0.2
		Spleen	Control	38	2.6
		Spleen	2.4G2 IgG	7	0.4
C3H/HeN	k	Peritoneal	Control	38	2.7
		Peritoneal	2.4G2 IgG	2	0.1
		Peritoneal	2.4G2 Fab	7	0.3
BALB/c	d	Peritoneal	Control	72	6.3
		Peritoneal	2.4G2 IgG	13	0.6
		Peritoneal	2.4G2 Fab	19	1.5
		Spleen	Control	44	2.9
		Spleen	2.4G2 IgG	21	1.0
		Spleen	2.4G2 Fab	16	0.7
NCSR	—	Peritoneal	Control	16	1.1
		Peritoneal	2.4G2 IgG	2	0.1
		Spleen	Control	40	2.4
		Spleen	2.4G2 IgG	4	0.3

Protocol for rosette formation was as detailed in Table II.

on cells of the H-2d haplotype (because the J774 cells used for immunization of the rat are of BALB/c origin) peritoneal cells and spleen cells from a number of different inbred mouse strains were screened. The results (Table III) show that ~40% of spleen cells from all strains tested formed EIgG rosettes, which is in agreement with previous results (35–36). The percentage of E₀ₓIgG rosetting cells in the resident peritoneal cell population was more variable, but preincubation with 2.4G2 IgG inhibited formation of E₀ₓIgG rosettes in all cases in both spleen cells and resident peritoneal cells. I attempted to screen for mouse neutrophil Fc receptor inhibition but could not obtain satisfactory rosetting in controls using cells from mouse peripheral blood, or from mouse peritoneal cavity 17 h after injection of 1 ml of thioglycollate medium.

Fc Receptors on Cells of Other Species. Peritoneal cells from rabbit, guinea pig, and rat, as well as human peripheral blood mononuclear cells and granulocytes, were tested to see if the 2.4G2 IgG inhibited binding of EIgG. Preincubation with the hybridoma antibody did not block EIgG rosetting on any of these cells. There was some binding over background of [125]I-2.4G2 IgG to guinea pig peritoneal cells, but this appeared to be Fc domain-mediated because there was no binding of the ^{125}I-Fab fragment. Thus, the antigenic site recognized by the hybridoma antibody is specific for a mouse determinant.

Discussion

The striking ability of macrophages and some lymphocytes to bind erythrocytes coated with IgG is a result of the presence of Fc receptors on the plasma membranes of these cells. These receptors confer specificity in the phagocytosis of antibody-coated

particles by macrophages (37), and in the destruction of target cells by lymphoid K cells and macrophages (38) in the presence of an appropriate anti-target cell antibody. The function of the FcR on B lymphocytes is not clear, but the interaction of complexes with FcR may be involved in regulation of the immune response (1-3).

The development of the technique for making monoclonal antibodies (16) has opened exciting possibilities for dissection of immunogenic cell surface components, and I felt this approach might be fruitful for studying FcR. The search for a monoclonal anti-FcR antibody was based on the assumption that such an IgG would block the binding of EIgG2b which were used in the screening assay. There have been reports of inhibition of Fc receptors on B cells by IgG and F(ab')$_2$ fragments directed against a variety of surface markers including Ia, H-2D, H-2K, Ly4.2, and mouse immunoglobulin (see Halloran and Schirrmacher [39] for review). Inhibition of mouse macrophage FcR by Fab or F(ab')$_2$ fragments of anti-mouse macrophage IgG has also been reported (40). Because it is unlikely that all of these allergeneic and xenogeneic sera are directed against FcR, it appears more likely that inhibition is a result of steric blockage of the receptors or to perturbation of the membrane in an as yet undefined manner. A series of monoclonal antibodies I have isolated, which are directed against various macrophage surface determinants, have no inhibitory effect on either FcR or the C' receptor, suggesting that the polyvalent character of the allogeneic and xenogeneic sera mentioned above may be an important factor in their ability to inhibit FcR.

The evidence that the monoclonal IgG secreted by the 2.4G2 hybridoma has an anti-FcR antigenic specificity rests in part on the binding of the antibody to different cell types. A series of independently isolated variants of the J774 macrophage-like cell line which were unable to bind EIgG2b (17) bound 5% as much 2.4G2 IgG as the parent clone bound. Also, all cells which failed to form E_{ox}IgG rosettes (L-cells, TLX-9, P3U1, L5178Y, EL-4, and thymocytes) failed to bind ^{125}I-2.4G2 in a quantitative and sensitive binding assay. Conversely, cell lines which did form E_{ox}IgG rosettes (P338, P388D$_1$, J774, PU5.1, and WEHI-231) bound ^{125}I-2.4G2 IgG. Mixed cell populations from mouse peritoneal cavity or spleen, after preincubation with 2.4G2 IgG, did not form rosettes with E_{ox}IgG. The inhibition of FcR on a variety of different cell types, from different H-2 backgrounds, and the absence of the antigen-recognized by the hybridoma antibody on FcRII negative mutants of a macrophage cell line both suggest strongly that the 2.4G2 IgG recognizes an antigenic determinant on FcRII.

The inhibition of binding of EIgG2b and EIgG1, but not EIgG2a by preincubation of macrophages with the Fab fragment of the hybridoma antibody demonstrates that the two macrophage Fc receptors are different antigenically. The result is consistent with the report by Diamond et al. (14) that binding of EIgG2a and EIgG2b is inhibited only by the homologous subclass of aggregated mouse IgG, but is not in agreement with Heusser et al. (13), who concluded that the trypsin resistant receptor bound aggregated antibody of all mouse subclasses. The inhibition of IgG2a and EIgG2a binding by preincubation of macrophages with the intact hybridoma antibody is apparently a result of immune blockade of the FcRI site by the hybridoma IgG Fc domain.

An important result of these experiments is the definition of a common determinant on macrophages and some lymphoid cells. The relative ease with which rabbit IgG-

coated E (which bind via both FcRI and FcRII [14, 17]) form rosettes on macrophages compared to lymphoid cells is probably a result of the presence of FcRI on macrophages and possibly to a higher density of FcRII sites. Lymphoid cells which bind monomeric IgG2a poorly (13) may not express FcRI. One of the earliest studies of B cell, FcR demonstrated an inhibition of binding of mouse anti-fowl IgG ^{125}I-fowl IgG complexes by IgG1 and IgG2b, but not IgG2a (41). Heusser et al. (13) found that monomeric IgG2a did not bind to lymphocytic lines and that the order of binding of aggregated mouse subclasses of IgG was IgG1 = IgG2b > IgG2a. Many binding studies have been performed with heat or bis-diazotized benzidine-induced aggregates, and it is not altogether clear that such large aggregates bind in similar fashion to immune complexes. The specificity of FcRII for IgG1 and IgG2b complexes, but not IgG2a complexes, appears to be the same as the specificity of FcR on mouse B cells.

There are numerous reports of the presence of FcR on some cells of T-lymphocyte origin (42–44). Anderson and Gray (44) found that 20–40% of thymocytes bound ^{125}I-mouse IgG2b aggregates by autoradiography. Similar results were reported by Stout and Hertzenberg (43) who reported 10% of thymocytes were FcR^{+} by use of fluorescinated keyhole limpet hemocyanin (KLH)-anti-KLH complexes and the fluorescence activated cell sorter. Basten et al. (42) could demonstrate thymus cell FcR only with aggregated human IgG and not with immune complexes or myeloma protein aggregates. In the present study, thymocytes did not form E$_{ox}$IgG rosettes, nor did they bind 2.4G2 IgG. However, there is no doubt that there are Thy-1 positive lymphoid lines which bear FcR (13, 45, 50), and it is possible that a subpopulation of T cells in the spleen have FcR which bear the 2.4G2 antigenic determinant. Cell fractionation studies are in progress to resolve this question.

Studies are planned to isolate and characterize the antigen recognized by the 2.4G2 IgG and to reconstitute, if possible, the FcRII molecule in lipid micelles. The antibody will also aid in understanding the role of FcR in the biological function of cells which bear the determinant.

Summary

To investigate the antigenic relationship between the macrophage and lymphocyte Fc receptors (FcR), a monoclonal antibody capable of blocking mouse macrophage Fc receptors was selected. Hybrids were formed by fusing the P3U1 mouse myeloma and spleen cells from a rat immunized with the mouse macrophage-like cell lines J774 and P388D₁. The Fab fragment of the monoclonal IgG secreted by clone 2.4G2, inhibited the trypsin-resistant Fc receptor II (FcRII), which is specific for immune aggregates of mouse IgG1 and IgG2b, but had no inhibitory effect on the trypsin-sensitive Fc receptor I (FcRI), which binds monomeric IgG2a and erythrocytes coated with IgG2a. Thus, the monoclonal 2.4G2 IgG appeared to be specific for macrophage FcRII.

Further evidence that the 2.4G2 IgG was directed against FcRII came from binding studies of the monoclonal antibody to J774 cells and a series of independently isolated variants which do not express FcRII. These variants of J774 bound 5% as much of the monoclonal antibody as the parent line, which bound 600,000 molecules of 2.4G2 IgG per cell.

The antigenic relatedness of mouse lymphocyte FcR to mouse macrophage FcRII was demonstrated by the binding of 2.4G2 IgG to FcR-bearing lymphoid cell lines

and the inhibition of the lymphocyte FcR by the monoclonal antibody. Preincubation of spleen cells and peritoneal cells with 2.4G2 IgG likewise inhibited rosette formation with ox erythrocytes coated with rabbit IgG. The ability of the hybridoma IgG to inhibit mouse FcRII was independent of the major histocompatibility complex. The 2.4G2 IgG antigenic determinant was not present on rat, guinea pig, rabbit, or human FcR-bearing cells.

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