

PURIFICATION OF A FUNCTIONAL MOUSE Fc RECEPTOR THROUGH THE USE OF A MONOCLONAL ANTIBODY*

BY IRA S. MELLMAN‡ AND JAY C. UNKELESS§

From the Department of Cellular Physiology and Immunology, The Rockefeller University, New York 10021

Receptors for the Fc domain of IgG (Fc receptors [FcR]¹) have been found on a variety of macrophage and lymphoid cell types (1). FcR activity of mouse macrophages has been the most extensively studied, and current evidence indicates the presence of two distinct receptors. Based on quantitative binding data, several laboratories have concluded that both IgG1 and IgG2b bind to a trypsin-resistant site, whereas IgG2a binding is sensitive to prior trypsinization (2-7). In addition, aggregated IgG2b was found not to inhibit the binding of IgG2a, and vice versa (2-4). Further support for the two-receptor hypothesis comes from the analysis of FcR-negative derivatives of the macrophage-like cell line J774. Although the variant clones were almost totally deficient in the ability to bind IgG2b, they bound IgG2a normally (8).

Recently, we reported the isolation of a monoclonal rat anti-mouse FcR antibody that appears to be specifically directed against the trypsin-resistant IgG2b receptor (9). The Fab fragment of this antibody, designated 2.4G2 IgG, inhibited rosette formation by IgG1- and IgG2b-sensitized sheep erythrocytes (E) on mouse macrophages, neutrophils, and spleen cells as well as on mouse FcR-bearing cell lines of macrophage, B, T, and null lymphocyte origin. Rosette formation by IgG2a-coated E, on the other hand, was unaffected. Moreover, ¹²⁵I-2.4G2 Fab bound only to FcR-bearing cell types, and the binding of 2.4G2 to macrophages was unaffected by prior trypsinization of the cells.

It is clear that the further characterization of FcR activity requires the isolation and analysis of the receptor molecule(s). Accordingly, there have been a number of reports of the partial purification of FcR from both mouse and human macrophages and lymphocytes. Most of these studies have employed techniques of affinity chromatography with IgG immobilized on a solid support to isolate proteins from cell lysates. However, no consensus has emerged regarding even such a basic characteristic

* Supported by grants AI 14603, AI-07012, and Biomedical Research Support Grant 5-SO7-RR-07065 from the U. S. Public Health Service, National Institutes of Health.

‡ Fellow of the Leukemia Society of America, Inc., New York.

§ Andrew W. Mellon Foundation Fellow, New York.

Abbreviations used in this paper: BSA, bovine serum albumin; CNBr, cyanogen bromide; Con A, concanavalin A; DNP, dinitrophenyl; E, sheep erythrocytes; E_{TNP}, E that were derivatized with a trinitrophenyl moiety; E_{TNP} IgG, E_{TNP} that were coated with serial dilutions of affinity-purified rabbit anti-DNP IgG; FBS, fetal bovine serum; FcR, Fc receptor(s); HSA buffer, buffer that contained 0.6 M NaCl; 0.0125 M KPO₄, pH 7.4; and 0.02% NAN₃; K_a, association constant(s); M_r, apparent molecular weight; NP40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TNP, trinitrophenyl.

as the apparent molecular weight (M_r) of the molecule, with estimates ranging from 20,000 to 255,000 (10–18).

In this study, we have employed the anti-FcR antibody 2.4G2 as an affinity reagent to accomplish the one-step purification of the receptor to apparent homogeneity from the mouse macrophage cell line J774. 100- μ g quantities of the receptor glycoprotein were isolated and characterized biochemically. The purified material retained FcR activity in vitro, and the subclass specificity of the isolated FcR was examined.

Materials and Methods

Cell Culture. The J774 macrophage cell line, P388 lymphoma, PU 5.1 null cell line, and the WEHI 231 B cell line were maintained as described previously (9). The S49.1 T cell line, P815 mastocytoma, and RAW 309 Cr.1 macrophage cell line (all obtained from the Cell Distribution Center, Salk Institute, La Jolla, Calif.) were grown in α -modified Eagle's medium (KC Biological, Inc., Lenexa, Kans.) supplemented with 5% or 10% fetal bovine serum (FBS; heat inactivated at 56°C for 30 min; Flow Laboratories, Inc., Rockville, Md.); 100 U/ml penicillin, and 100 μ g/ml streptomycin. Peritoneal macrophages were obtained by lavage from CD₂F₁ mice (Flow Laboratories, Inc.) 4 d after intraperitoneal injection of 1 ml thioglycolate broth, as described (19).

Macrophages were labeled with [³⁵S]methionine, [³⁵S]cysteine (>650 Ci/mmol, Amersham Corp., Arlington Heights, Ill.), or [³H]leucine (50 Ci/mmol, New England Nuclear, Boston, Mass.) by overnight incubation in methionine-, cysteine-, or leucine-free medium, respectively. Radioactive amino acids were added at 50–100 μ Ci/60-mm dish.

Monoclonal Antibodies. Monoclonal anti-dinitrophenyl (DNP) IgG1- and IgG2b-secreting hybridomas DHK 109.3 and DHK 10.12 were obtained through the generosity of Dr. Norman Klinman (Scripps Clinic and Research Foundation, La Jolla, Calif.). Ascites fluid from these tumors was prepared by injection of 5×10^6 cells into CAF₁ mice (The Jackson Laboratory, Bar Harbor, Maine) 1 wk after intraperitoneal injection of 0.5 ml of pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The antibodies were purified by affinity chromatography on Sepharose 4B coupled with DNP₁₂ bovine serum albumin (BSA; crystallized one-time; Sigma Chemical Co., St. Louis, Mo.) essentially as described previously (7). The anti-DNP IgG3 antibody 8-11-A was kindly given to us by Dr. Julian Fleischman and Dr. Mitchell Scott (Washington University School of Medicine, St. Louis, Mo.). Monoclonal IgG1 and IgG2b anti-sheep (E) antibodies SP/3 and SP/2 were from Accurate Chemical & Scientific Corp., Hicksville, N. Y. The monoclonal anti-E IgG2a, UN-2, was obtained through the generosity of Dr. Betty Diamond (Albert Einstein College of Medicine, Bronx, N. Y.).

The monoclonal rat anti-mouse FcR antibody 2.4G2 was prepared and purified as described previously (9).

Radiolabeling of Cell Surfaces. Plasma membrane proteins were radioiodinated using a slight modification of the lactoperoxidase-glucose oxidase method of Hubbard and Cohn (20) and Mellman et al. (21). Terminal galactose residues of cell surface glycoproteins were labeled by galactose oxidase oxidation followed by reduction with NaB[³H₄]. In some experiments, cells were preincubated with neuraminidase (22). Reagents were obtained as follows: lactoperoxidase (purified grade) and neuraminidase (B grade) from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; carrier-free Na[¹²⁵I] and NaB[³H₄] (10 Ci/mmol) from Amersham Corp.; galactose oxidase and glucose oxidase (type V) from Sigma Chemical Co.

Iodination of Proteins. Proteins (≤ 50 μ g) were iodinated in a vol of 0.1 ml of 0.1 M NaP_i (pH 7) that contained 0.9 μ g chloramine T and 0.5 mCi carrier-free Na[¹²⁵I] (23). After incubation at 4° for 30 min, 10 μ l 1 M KI was added and the entire reaction mixture was passed over a 0.3-ml column of Dowex 1 \times 8 Cl⁻ (Dow Chemical Co., Midland, Mich) (in phosphate-buffered saline [PBS] with or without 1 mg/ml BSA) to remove free iodide. FcR was iodinated similarly except that, because the FcR was bound by the resin, free iodide was removed by dialysis against PBS that contained a small quantity of Dowex 1 \times 8 Cl⁻. Adsorption of the iodinated FcR to glass or plastic was minimized by using tubes precoated with 2% FBS and then rinsed thoroughly with water.

Immune Precipitation. Labeled cells (0.5×10^7 – 3.0×10^7) were solubilized in lysis buffer (PBS

that contained 0.5% Nonidet P-40 [NP40; Bethesda Research Labs., Bethesda, Md.], 0.2 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma Chemical Co.] and nuclei and debris were removed by centrifugation (45,000 *g*, 15 min at 4°C). 1 µg of 2.4G2 Fab was added to 0.2 ml of clarified supernate (prepared from 0.5×10^7 – 1×10^7 cells) and incubated for 1 h on ice. Antibody-antigen complexes were recovered using 50 µl of a 50% suspension of affinity-purified F(ab')₂ fragments of rabbit anti-rat IgG (N. L. Cappel Laboratories, Cochranville, Pa.) coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (21). Comparable results were obtained by adding 50 µl of a 50% suspension of 2.4G2 Fab-Sepharose 4B directly to the cell lysate. In either case, the immunoadsorbent was incubated at 4°C with constant agitation for 1 h, collected by centrifugation, washed twice in buffer that contained 0.6 M NaCl; 0.0125 M KPO₄, pH 7.4; and 0.02% NaN₃ (HSA buffer), twice at room temperature with a mixed detergent solution (24) (0.05% NP40; 0.1% sodium dodecyl sulfate [SDS]; 0.3 M NaCl; and 10 mM Tris-HCl, pH 8.6), and once again with HSA buffer. The antigen was then eluted in 40 µl of electrophoresis sample buffer (2% SDS; 12% sucrose; 0.01% bromphenol blue; 50 mM dithiothreitol; and 50 mM Na₂CO₃ buffer, pH 8.6) by boiling for 2 min.

Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE was performed according to the method of Neville et al. (25) using 1-mm-thick slab gels with a 4–11% polyacrylamide gradient in the running gel. For samples that contained ³H or ³⁵S, gels were processed for fluorography as described (26) and exposed at –70°C on prefogged Kodak RP X-Omat film. ¹²⁵I-containing lanes were dried and exposed on Kodak RP X-Omat or Du Pont Cornex film using image-intensification screens (Lightning Plus; E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.).

Two-dimensional PAGE was performed using a modification (27) of the original procedure described by O'Farrell (28). To look for proteins with basic isoelectric points, some gels were run under nonequilibrium conditions (27). The pH gradient in equilibrium focusing gels was determined by elution of ampholytes from individual gel slices in water. Gels were dried and exposed as described above.

Assay for Solubilized FcR. The concentration of FcR in solution was estimated by the ability of the solubilized antigen to bind ¹²⁵I-2.4G2 Fab. Small aliquots (≤20 µl) of the sample to be tested were added at various dilutions to a 1.5-ml Eppendorf microfuge tube (Brinkmann Instruments, Inc., Westbury, N. Y.) that contained lysis buffer (50 µl) and 0.05 µg ¹²⁵I-2.4G2 Fab (~1.5 × 10⁶ cpm). After 15 min on ice, the reaction mixture was applied to a 0.25-ml column of concanavalin A (Con A) (Sigma Chemical Co.) coupled to CNBr-activated Sepharose 4B (29). The column was then washed four times with 1-ml aliquots of PBS at room temperature to remove unbound radioactivity, and then eluted twice with 1-ml portions of 1% SDS. Because the FcR is a glycoprotein, FcR-¹²⁵I-2.4G2 Fab complexes that formed were bound by the column, whereas free ¹²⁵I-2.4G2 Fab (which lacks carbohydrate) was eluted by the PBS washes. ¹²⁵I was determined using a Packard Auto-Gamma Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). In each experiment, background radioactivity was determined by including duplicate tubes to which FcR-containing samples had not been added, or that contained FcR that had been preincubated with a large excess of unlabeled Fab. Both methods yielded equivalent background binding. The assay reached a plateau when 20–25% of the input ¹²⁵I-2.4G2 Fab was bound; previous results demonstrated that 50–60% of the ¹²⁵I-2.4G2 Fab could be adsorbed specifically by J774 cells (9). Thus it is possible that a subpopulation of FcR was not detected by this assay. Supporting this, the amount of FcR measured in lysates was approximately one-third of that detected on an equal number of cells by binding of ¹²⁵I-2.4G2 Fab. One unit of FcR was defined as the amount of antigen that bound 1 µg of 2.4G2 Fab.

Purification of Macrophage FcR. The antigen recognized by 2.4G2 IgG was purified from J774 cells grown either in suspension culture or as subcutaneous tumors in syngeneic CD₂F₁ mice (Flow Laboratories, Inc.). Cell pellets and tumors were stored for up to 3 mo at –70°C before use. Lysates were prepared by homogenizing pellets or tumors in cold lysis buffer with a Potter-Elvehjem homogenizer. The homogenates were clarified by centrifugation, first at 1,600 *g* (5 min at 4°C) to remove nuclei, and then at 40,000 *g* (30 min at 4°C). The final supernate was removed carefully to minimize contamination by floating lipids. The A₂₈₀ of the lysates ranged from 30 to 65/ml, and up to 35 ml of lysate was employed in any individual isolation. To

facilitate calculation of recoveries, a trace amount of [³⁵S]methionine-labeled J774 lysate ($>4 \times 10^7$ cpm from $\sim 10^7$ cells) was added at the homogenization step.

Lysates were then batch-adsorbed with 2.4G2 Fab coupled to CNBr-activated Sepharose 4B for 1 h at 4°C with continuous agitation. Sufficient resin was added (equivalent to ~ 0.2 mg 2.4G2 Fab/ml of lysate) to remove $>90\%$ of the antigen from solution, measured as described above. The immunoadsorbent was washed with the same sequence of buffers as was used for the immunoprecipitations. To elute the antigen from the resin, a high-pH elution scheme similar to that used by McMaster and Williams (30) was employed. The immunoadsorbent was packed into a small column, equilibrated with 0.5% deoxycholate in 0.1 M Tris-HCl at pH 7.9, and the antigen eluted with successive column volumes of PBS that contained 0.5% deoxycholate and 0.1 M triethylamine, pH 11.5. The eluates were immediately neutralized by collection into tubes containing 1 M Tris-HCl, pH 7.4 (20% of the column volume). The recoveries using this method were higher than those obtained by elution with 3 M ammonium thiocyanate, 8 M urea, 1 M propionic acid, or 0.5 M acetic acid. [³⁵S]methionine-labeled proteins and antigenic content were determined in individual fractions. Peak tubes were pooled and dialyzed overnight against two 500-*vol* of PBS that contained 0.02% NaN₃ and stored at 4°C, or against one 250-*vol* of 0.1 M (NH₄)₂CO₃ and lyophilized. Antigenic activity was not destroyed as a consequence of these procedures. Protein was determined according to the method of Lowry et al. (31) or by using a Coomassie blue binding assay (32).

Preparation of Membranes from J774 Cells. J774 cells (5×10^8) were homogenized in 15 ml of cold 25 mM Hepes, pH 7.6 (which contained 0.2 U/ml aprotinin and 1 mM PMSF), with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000 *g* (1 h at 4°C). The pellet was resuspended by brief sonication in the same Hepes buffer that contained 5 mM MgCl₂ and treated with deoxyribonuclease I (50 μg/ml; Worthington Biochemical Company, Freehold, N. J.) for 30 min on ice. Membranes were then washed by three successive centrifugations after resuspension in homogenization buffer.

¹²⁵I-FcR Binding to Protein-coated Particles. Superfine Sephadex G-25 (10–40 μm diameter) was activated using CNBr at a ratio of 200 mg CNBr:g wet weight of Sephadex as described previously (7). Activated beads were incubated with proteins (5 mg/ml DNP₁₂BSA or 5 mg/ml BSA per g Sephadex; 1 mg/ml LPC-1 or 1 mg/ml 2.4G2 Fab per g Sephadex) overnight with agitation at 4°C in PBS that contained 50 mM NaHCO₃, pH 8.7. The amount of LPC-1, rabbit anti-DNP IgG, and 2.4G2 Fab bound (determined by radioactivity bound from a trace amount of ¹²⁵I-protein added in the initial incubation) was 300 μg, 270 μg, and 120 μg/g Sephadex, respectively. DNP₁₂BSA-Sephadex beads were coated with anti-DNP IgG or F(ab')₂ fragment by incubation at room temperature for 15 min with frequent agitation. Essentially all the IgG or F(ab')₂ fragment added bound to DNP₁₂BSA Sephadex, up to a concentration of 200 μg protein/ml packed Sephadex. On the other hand, BSA-Sephadex beads bound $<0.2\%$ of the input ¹²⁵I-packed rabbit anti-DNP IgG.

All binding studies of ¹²⁵I-FcR were performed in PBS that contained 1–2% FBS. The addition of FBS did not affect the specific binding, but had a marked effect on lowering nonspecific binding to the Eppendorf centrifuge tubes. BSA was not an effective substitute for FBS. ¹²⁵I-FcR was added to 5–10 μl of coated Sephadex beads in a total vol of 50 μl, incubated at room temperature for 15–30 min, and the Sephadex was then rinsed with three 1-ml washes of PBS that contained 1% FBS. Bound radioactivity was assayed in a Packard gamma counter. Similar procedures were followed for binding of ¹²⁵I-FcR to IgG-coated erythrocytes.

To test the binding of ¹²⁵I-FcR as a function of pH, DNP₁₂BSA Sephadex beads coated with rabbit anti-DNP IgG or its F(ab')₂ fragment were equilibrated in buffer (50 mM acetate; 50 mM phosphate; 50 mM borate) that contained 1% FBS adjusted to desired pH values with NaOH. The binding assay contained 30 μl of buffer, 5 μl of ¹²⁵I-FcR diluted 1:10 in 0.15 M NaCl (2.5×10^{-3} U, 1×10^4 cpm), and 10 μl of coated Sephadex beads. After incubation, tubes were rinsed twice with 1-ml portions of the same pH buffer used in the incubation, and assayed for bound radioactivity. To test for renaturation, FcR adjusted to pH 4 in the above buffer and kept at room temperature for 30 min was then neutralized with 1 M Tris base before assay as usual. The ¹²⁵I-FcR-binding activity was unaffected by digestion with 80 μg/ml phospholipase C (from *Bacillus cereus*; grade II; C. F. Boehringer and Sons, Mannheim, Federal Republic of Germany) for 30 min at 37° in PBS that contained 1.5 mM CaCl₂ and 10 mM MgCl₂.

Binding of ^{125}I -Proteins to Intact Cells. Binding of ^{125}I -proteins to cells was performed as described previously (6-9). Cells (2×10^5 - 4×10^5) plated in 16-mm wells (Costar, Data Packaging, Cambridge, Mass.) were incubated with 0.3 ml of ^{125}I -protein in PBS that contained 1 mg/ml BSA for 1 h at 4°C. The radioactive mixture was then rapidly aspirated and the trays were dipped into two successive beakers filled with 2 liters of cold PBS. Excess fluid was again aspirated, and the wells swabbed with Q-tips (Chesebrough-Ponds, Inc., Greenwich, Conn.) which were then assayed for radioactivity. Nonspecific (nonsaturable) binding was assessed by binding of ^{125}I -protein of low specific activity. All determinations were performed in triplicate and experimental values did not differ by >10%.

The dissociation of bound ^{125}I -2.4G2 Fab from J774 cells was measured in the presence of 25 μg of unlabeled 2.4G2 Fab. Pseudo-first-order association constants were determined for concentrations of ^{125}I -Fab from 2×10^{-8} to 8×10^{-8} M. These constants were plotted against concentration to obtain the rate of association.

Results

Antigen on Murine Cells and Cell Lines Recognized by 2.4G2 IgG. Because FcR have been described on a variety of lymphoid cell lines and cell types, it was of interest to compare the antigens recognized by the monoclonal anti-mouse FcR antibody 2.4G2 on representative FcR-bearing cells. Two immune precipitation systems were used with equivalent results. Labeled cell lysates were incubated either with 2.4G2 Fab (or IgG) coupled to Sepharose 4B or with free 2.4G2, after which, immune complexes were isolated using Sepharose 4B coupled with rabbit anti-rat IgG F(ab')₂ fragments (21).

As shown in Fig. 1, the iodinated antigens isolated from different cell types displayed significant heterogeneity in M_r after SDS-PAGE, ranging from 70,000 (WEHI 231 B cell line) to 47,000 (thioglycollate-elicited mouse peritoneal macrophages). The antigen isolated from the J774 macrophage cell line had two components, a major band centered at 60,000 M_r and a minor band at ~47,000 M_r . No other low molecular weight components were consistently observed. Electrophoretic patterns were similar when SDS-PAGE was carried out under reducing and nonreducing conditions, indicating the absence of extensive interchain disulfide linkages.

The breadth of the radioactive bands in Fig. 1 is also noteworthy, with a spread of up to 10,000 M_r . This observation suggested that the antigen recognized by 2.4G2 Fab was glycosylated. To test this hypothesis, immune precipitation was performed using NP40 lysates of J774 cells previously labeled by $\text{NaB}[^3\text{H}]_4$ reduction after galactose oxidase treatment, a procedure that results in the tritiation of terminal galactose residues (22). As shown in Fig. 2, radiolabeled antigen was detected, indicating the presence of carbohydrate. More radioactivity was incorporated when the cells were treated with neuraminidase before galactose oxidase, suggesting that sialic acid was also present. The antigen could also be labeled metabolically with [^{35}S]methionine (Fig. 2), [^{35}S]cysteine, and [^3H]leucine (data not shown).

Trypsin treatment of J774 cells (1 mg/ml of trypsin for 30 min at 37°C) did not reduce the binding of ^{125}I -2.4G2 (data not shown) but resulted in a slight shift of FcR to lower M_r . In immune precipitates from lysates of cells iodinated after trypsinization, the lower M_r species of the two J774 bands appeared to be more prominent (Fig. 2).

Assay for Soluble FcR. Before attempting to isolate the FcR in larger amounts, we developed a quantitative assay for the antigen in solution to enable us to monitor the purification. The assay was based on the ability of Con A-Sepharose to retain ^{125}I -2.4G2 Fab-FcR complexes while not binding free ^{125}I -2.4G2 Fab (which lacks carbo-

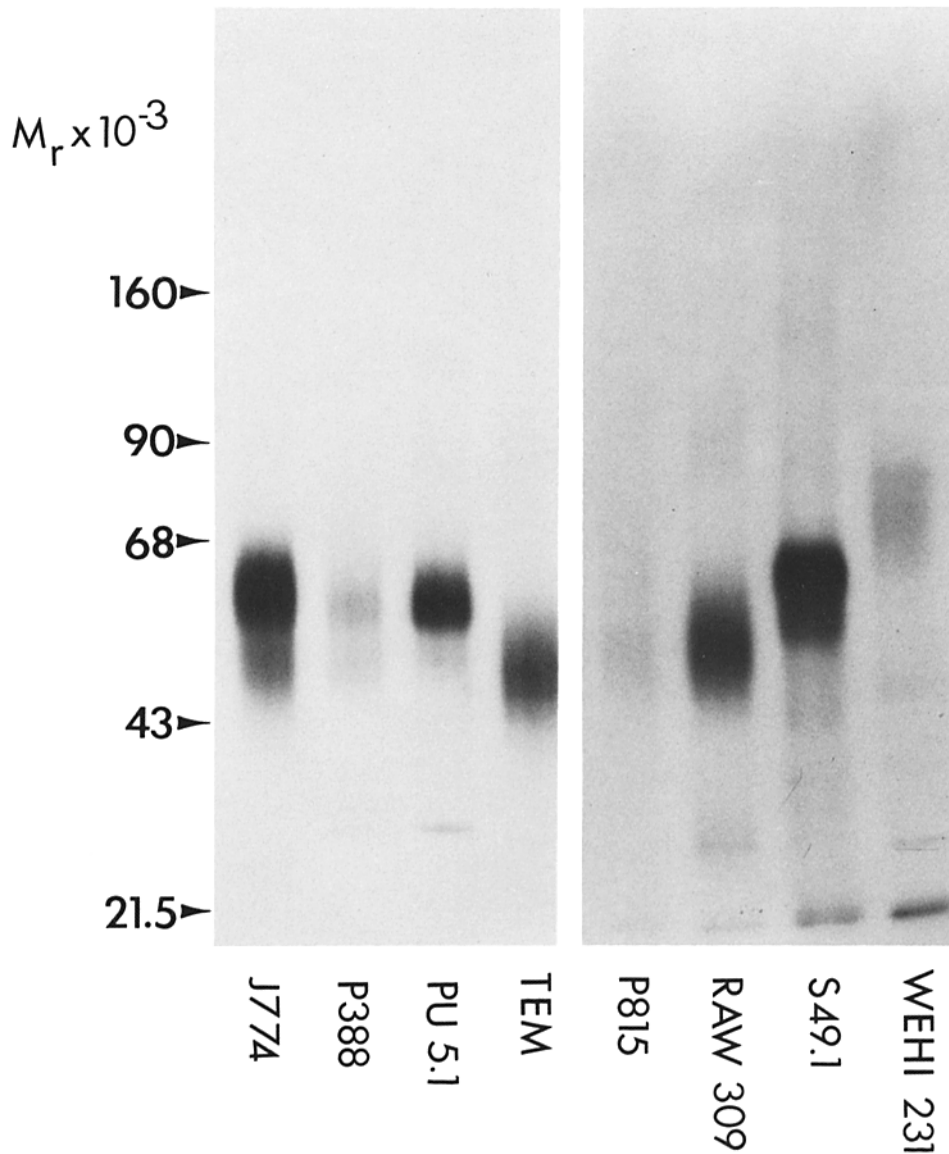


FIG. 1. Antigens recognized by 2.4G2 on different FcR-bearing cell types. Cells were iodinated using lactoperoxidase-glucose oxidase and then lysed in NP40. Immune precipitations were performed as detailed in Materials and Methods. No radioactive bands were detected when 2.4G2 was omitted. From left to right the cell types shown are: J774 (macrophage), P388 (null lymphocyte), PU 5.1 (null lymphocyte), thioglycollate-elicited macrophage (TEM), P815 (mastocytoma), RAW 309 Cr.1 (RAW 309) (macrophage), S49.1 (T lymphocyte), and WEHI 231 (B lymphocyte). No antigen was detected in iodinated NP40 lysates of the FcR-negative derivative of J774, MNNG 6.31 (15).

hydrate). Aliquots of the samples to be tested were incubated with ^{125}I -2.4G2 Fab until equilibrium was reached (15 min at 0°) and then passed over small columns of Con A-Sepharose. The radioactivity specifically retained by these columns was linear

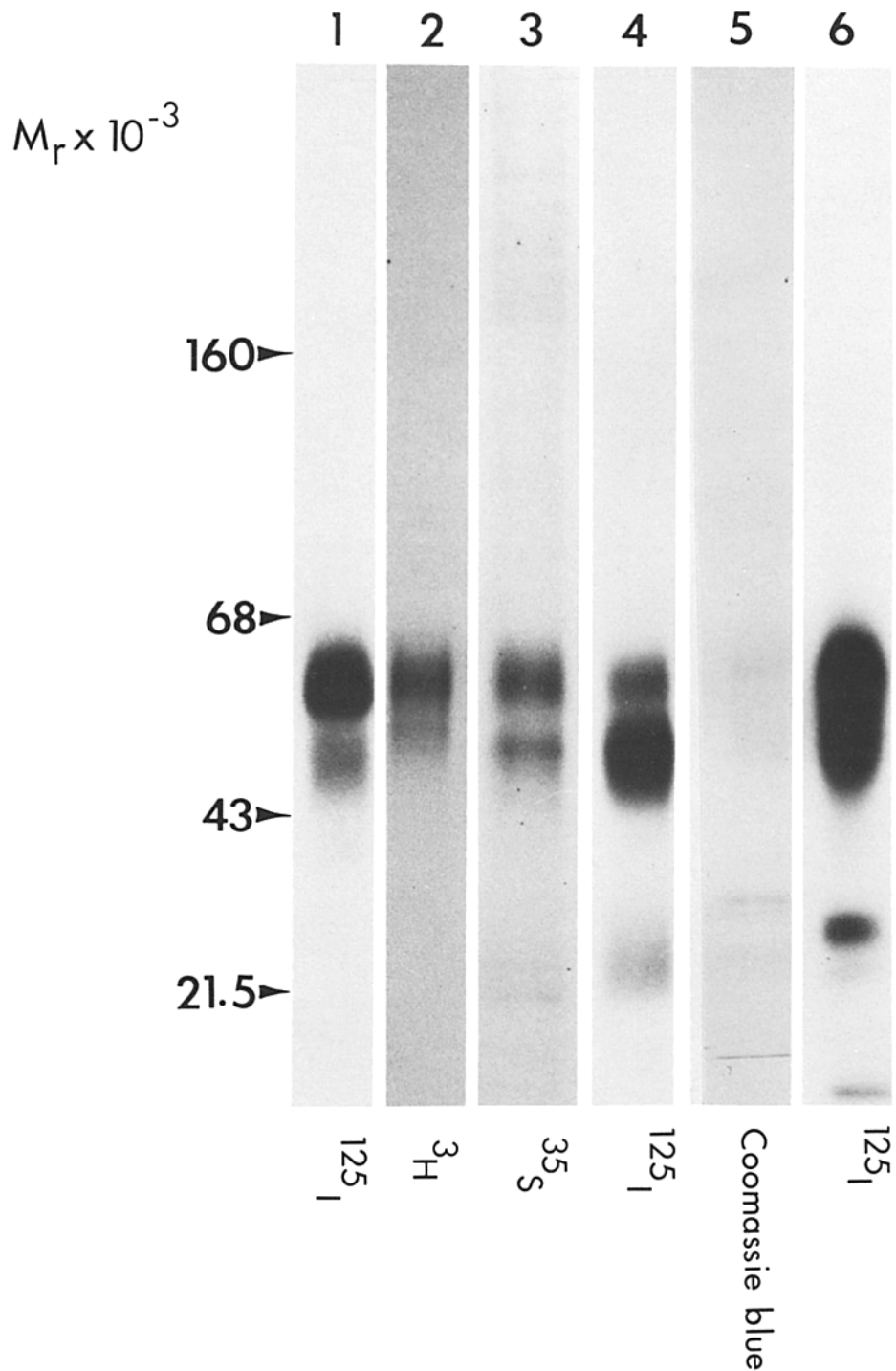


FIG. 2. Characterization of the antigen recognized by 24G2 Fab on J774 macrophages. Lanes 1 and 4: immune precipitation from J774 cells. Lanes 1 and 4, J774 cells labeled by lactoperoxidase-glucose oxidase catalyzed iodination; lane 2, cells labeled by galactose oxidase followed by reduction with NaB^{3}H_4 ; lane 3, cells labeled by overnight incubation in ^{35}S methionine-containing medium. The cells in lane 4 were treated with 1 mg/ml of trypsin (15 min at 37°C) before iodination. Lane 5, antigen isolated from J774 tumors and stained with Coomassie brilliant blue. Lane 6, antigen isolated from J774 tumors, iodinated in vitro with chloramine T.

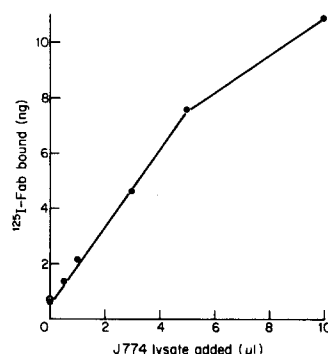


FIG. 3. Con A-Sepharose assay for solubilized FcR. J774 cells (8.7×10^7) were vortexed in 1 ml of lysis buffer. After centrifugation to remove nuclei and debris, ~ 28 mg of protein remained in the supernate. Aliquots of lysate were added to 50- μ l portions of lysis buffer, mixed with ^{125}I -2.4G2 Fab (50 ng; 1.1×10^6 cpm/ μ g), and processed as described. The number of FcR sites assayed in this experiment was 1.9×10^6 sites/cell, somewhat lower than the saturation binding of ^{125}I -2.4G2 Fab to the intact cells, which was 6×10^6 sites/cell (9). Background binding was not subtracted. (○) Binding after 10 μ l of J774 lysate was preincubated with 10 μ g of 2.4G2 Fab.

TABLE I
Purification of FcR from J774 Tumors by Affinity Chromatography with 2.4G2 Fab-Sepharose

	Protein*	[³⁵ S]Methionine	FcRdd	Specific activity
	mg	cpm	U	U/mg protein
NP40 homogenate§	1337.7	3.36×10^7	139	0.1
Column eluate	0.149	1.38×10^4	79	530.2
(Recovery)	(0.011%)	(0.041%)	(57%)	—

§ The initial homogenate was centrifuged (1,600 g for 5 min) to remove nuclei and debris; the resulting supernate was designated NP40 homogenate. The protein and [³⁵S]methionine values of the initial homogenate were approximately twofold greater.

* Protein was determined using the Coomassie blue binding assay (32).

‡ Determined as described in Materials and Methods. 1 U is ~ 1 μ g FcR.

with respect to the amount of protein added (Fig. 3); 1 U of FcR was defined as that amount of antigen capable of binding 1 μ g of 2.4G2 Fab.

Purification and Biochemical Characterization of the FcR. The procedure developed for the large scale purification of the FcR was fundamentally the same as the immune precipitation protocol described above. NP40 lysates (which contained 1–2 g cell protein) were prepared from subcutaneous J774 tumors. After batch-adsorption with 2.4G2 Fab-Sepharose, the resin was washed extensively, and bound antigen was then eluted with 0.1 M triethylamine (pH 11.5) in 0.5% deoxycholate (30) (Materials and Methods).

A typical purification, in which a lysate of [³⁵S]methionine-labeled J774 cells was added to the tumor lysate to facilitate calculation of yields, is shown in Table I. The overall recovery of FcR in different experiments varied from 40–80%, with specific activities of the isolated material $>5,000$ -fold greater than the initial lysate, cleared of nuclei. This purification factor is consistent with the theoretical yield, because the percentage abundance of a 50,000-dalton protein (present at 500,000 molecules/cell [9]) should be $\sim 0.01\%$, given 300 μ g protein/J774 cell.

Sufficient antigen was isolated to be visualized by Coomassie blue staining after SDS-PAGE. As shown in Fig. 2, the electrophoretic profile of the purified material was essentially identical to that obtained after immune precipitation from lysates of iodinated or metabolically labeled J774 cells (Figs. 1 and 2). No significant contamination by unrelated polypeptides was observed. The three low M_r bands visible just behind the electrophoretic front probably represent 2.4G2 light-chain and heavy-chain fragments dissociated from the CNBr-activated Sepharose by nucleophilic displacement during the high-pH elution step.

To facilitate study of the small amount of purified antigen, protein stripped from the affinity column was iodinated using chloramine T after dialysis against PBS. As shown in Fig. 2, the electrophoretic profile of the isolated material iodinated in this way was the same as that detected by Coomassie blue staining or by immune precipitation from lactoperoxidase-iodinated or [35 S]methionine-labeled cells. The 125 I-labeled protein was further characterized by two-dimensional PAGE, in which the isoelectric-focusing dimension was performed under nonequilibrium conditions (27) to retain proteins with basic isoelectric points on the gel. As shown in Fig. 4A, there are again clearly two components of the J774 FcR, and a series of components of low M_r , which probably are contaminating 2.4G2 light- and heavy-chain fragments. Digestion of the 125 I-FcR preparation with neuraminidase (Fig. 4B) resulted in a shift of the 47,000 and 60,000 M_r components to more basic positions relative to the low M_r peptides, which strengthens the conclusion that the FcR contains sialic acid.

To estimate the M_r of the isolated FcR in aqueous solution, a sample of the iodinated material was first subjected to gel filtration on a column of Sephadex G-

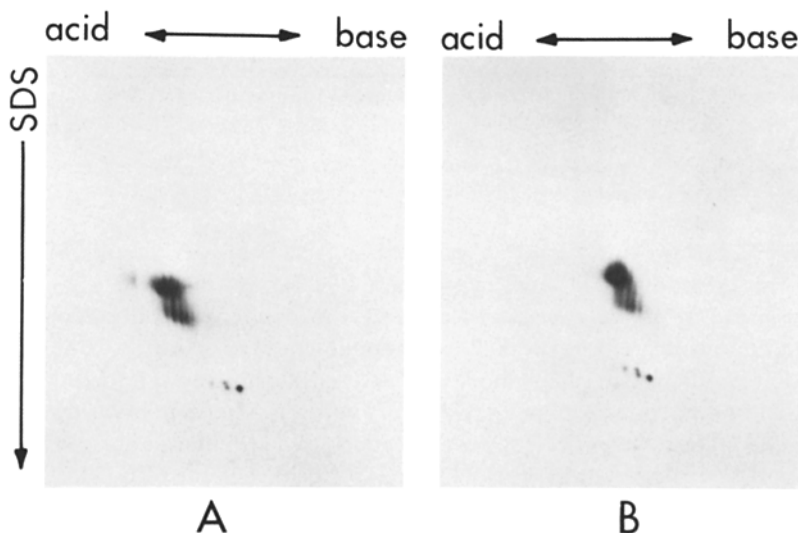


FIG. 4. Analysis of the isolated 2.4G2 antigen by two-dimensional PAGE. 2.4G2 antigen, purified from J774 tumors and iodinated in solution using chloramine T, was analyzed by two-dimensional electrophoresis (26) and visualized by radioautography. Isoelectric focusing (horizontal dimension) was performed under nonequilibrium conditions (27) to retain species with alkaline isoelectric points on the gel. (A) Iodinated material without further treatment. (B) Material digested with neuraminidase (50 U/ml at 37°C for 30 min). Note that neuraminidase treatment shifts the position of the major radioactive bands (toward the alkaline side of the gel) relative to a series of minor punctate spots of lower M_r . These low M_r spots probably represent contaminating 2.4G2 light- and heavy-chain fragments.

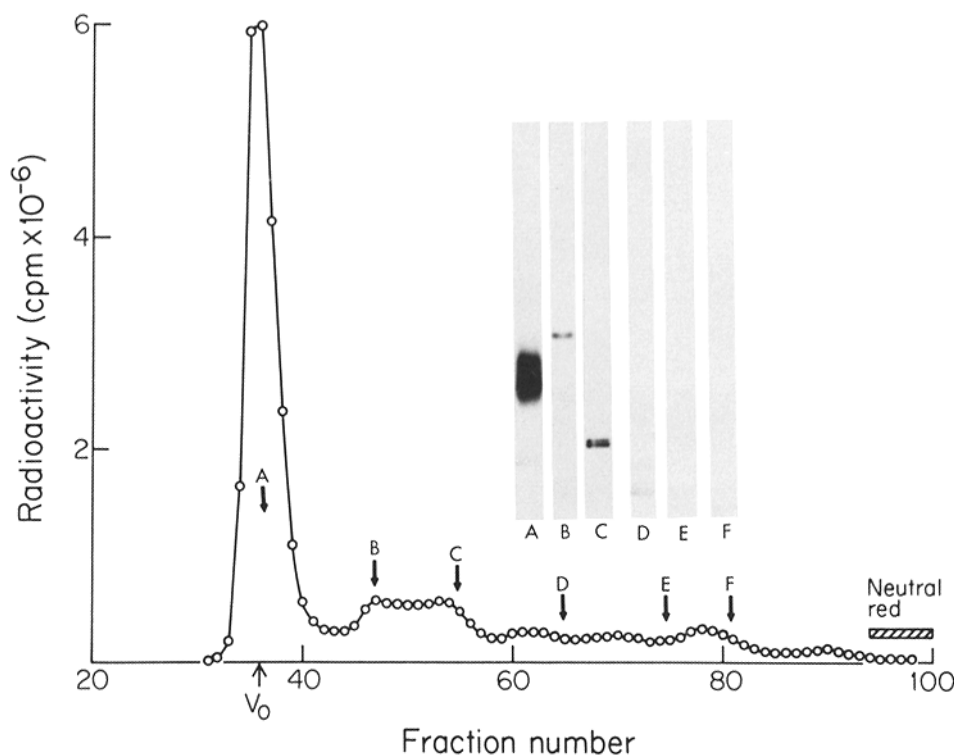


FIG. 5. Chromatography of purified ^{125}I -FcR on Sephadex G-150. Labeled FcR (5.9×10^7 cpm; 1 U) was applied to a 1.4×75 -cm column of fine Sephadex G-150 and eluted with 0.5 M NaCl - 50 mM NaPO_4 buffer at a flow rate of 3 ml/h. To minimize adsorption of labeled material, fractions were collected in glass tubes previously coated with 1% FBS and rinsed exhaustively with water. The recovery of radioactivity applied to the column was 60%. Of the initial ^{125}I -FcR, 35% bound to rabbit anti-DNP IgG-coated DNP₁₂BSA-Sephadex G-25 beads; control DNP₁₂BSA beads bound 0.7% of the input. Specific binding of iodinated proteins to IgG-coated beads was found only in the void-volume peak. IgG-coated beads bound 54% of the input radioactivity, the $\text{F}(\text{ab}')_2$ control was 0.3%, and the uncoated DNP₁₂BSA Sephadex bound 1.3% of the input.

150. Approximately 50% of the radioactivity eluting from the column was found in the void volume peak (Fig. 5), suggesting the presence of large aggregates. SDS-PAGE of column fractions confirmed that the protein previously identified as the FcR from surface iodinated cells was found only in the void volume.² The size of the FcR aggregates was then assessed by velocity sedimentation analysis in 5–20% sucrose gradients. The iodinated material exhibited a broad peak at $\sim 15\text{S}$, extending well past the IgM marker (Fig. 6). In addition, a variable amount of radioactivity (10–30%) sedimented to the 60% sucrose cushion. Thus, in aqueous solution, the isolated antigen formed high-order aggregates of at least 10–20 subunits.

Activity of the Isolated FcR. Circumstantial evidence based on the restriction of binding of 2.4G2 IgG to FcR-bearing cells, and the subsequent inhibition of rosette formation by EIgG on cells preincubated with 2.4G2 IgG, suggested the antigen

² In the experiment shown, nonspecific adsorption to the tubes was avoided by precoating with serum, which resulted in a slight contamination with serum albumin (see Fig. 5, lane B). This iodinated band was not observed in other experiments in which serum coating of the tubes was omitted.

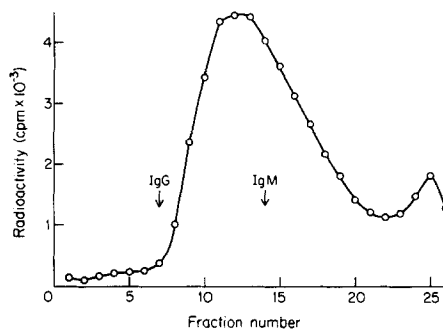


FIG. 6. Sucrose density velocity sedimentation of ^{125}I -FcR. Labeled FcR (4.7×10^4 cpm, ~ 0.01 U) was layered over a 5–20% sucrose gradient buffered with 50 mM Tris-HCl, pH 8, with a 1-ml cushion of 60% sucrose, and centrifuged in a Beckman SW-40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 8 h at 4°C . Fractions (0.5 ml) were collected and assayed for radioactivity. Companion gradients contained marker proteins LPC-1 (IgG) and a human IgM myeloma protein (courtesy of Dr. Shu Man Fu, The Rockefeller University, New York).

recognized by 2.4G2 IgG was an FcR. Using two different approaches, we then determined directly whether the isolated antigen exhibited FcR activity in vitro.

(a) *Hemagglutination Enhancement by FcR*. Because the isolated FcR formed large, presumably multivalent aggregates in solution, we reasoned that if it retained FcR activity, it should potentiate the agglutination of EIgG. E were derivatized with a trinitrophenyl (TNP) moiety (E_{TNP}) (33) and then coated with serial dilutions of affinity-purified rabbit anti-DNP IgG ($E_{\text{TNP}}\text{IgG}$) or its $F(\text{ab}')_2$ fragment. In the presence of as little as 0.1 U/ml FcR, the $E_{\text{TNP}}\text{IgG}$ formed large aggregates (within 15–30 min at room temperature) that could not be dispersed by vigorous vortexing. In contrast, E_{TNP} hemagglutinated only by the addition of rabbit anti-DNP IgG could be readily dispersed by such treatment (Fig. 7). This effect was specific for the Fc domain of the rabbit IgG; no enhancement of hemagglutination was observed when FcR was added to either E_{TNP} or E_{TNP} coated with an equivalent hemagglutinating titer of anti-DNP $F(\text{ab}')_2$. Even more-striking results were obtained with E coated with the IgG2b anti-E monoclonal SP/2 and the IgG2a anti-E monoclonal U88. These monoclonal antibodies did not by themselves cause hemagglutination and, thus, hemagglutination of E coated with SP/2 or U88 was observed only when FcR was added.

(b) *Binding of ^{125}I -FcR to Immobilized IgG*. To verify that the isolated antigen was binding to IgG, we assessed the interaction between the purified FcR and IgG immune complexes. For this purpose, IgG was immobilized on several different particles. First, we determined the extent to which ^{125}I -FcR bound to erythrocytes opsonized with mouse monoclonal IgG1, IgG2a, and IgG2b anti-E antibodies. As summarized in Table II, the iodinated material bound most effectively to IgG2b and IgG2a-coated E. Significant, but somewhat lower, amounts of ^{125}I -FcR bound to IgG1-coated E. The maximum binding observed was $\sim 50\%$ of the added radioactivity, whereas $<0.5\%$ of the radioactivity bound to uncoated E.

A second set of binding studies was performed using CNBr-activated Sephadex beads (7) to which either IgG2a (the mouse myeloma protein LPC-1), rabbit anti-DNP IgG, or DNP₁₂BSA was coupled. The DNP-BSA-Sephadex beads were in turn coated with antibody by incubation with rabbit anti-DNP IgG [or $F(\text{ab}')_2$] or any of

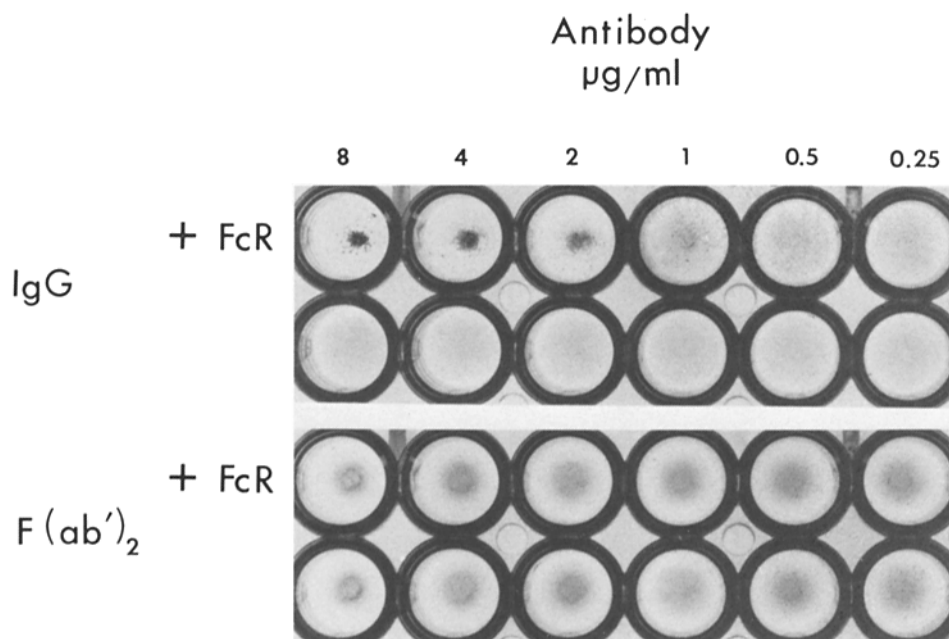


FIG. 7. Enhancement of hemagglutination by purified FcR. Erythrocytes ($50 \mu\text{l}$; 0.25% vol:vol in PBS that contained 2% FBS) derivatized with TNP groups as described by Rittenberg and Pratt (33) were incubated with anti-DNP IgG or $F(ab')_2$ fragment ($50 \mu\text{l}$) and allowed to stand for 2 h. The last hemagglutinating well contained $1 \mu\text{g}/\text{ml}$ of IgG or $F(ab')_2$ fragment. The cells were washed twice by centrifugation with $50 \mu\text{l}$ portions of PBS. Then, $10 \mu\text{l}$ of FcR (5 U/ml) in PBS or $10 \mu\text{l}$ were added, mixed by vortex, and allowed to stand at room temperature for 30 min with intermittent mixing. Finally, the trays were vortexed vigorously and photographed 10 min later.

a series of monoclonal anti-DNP antibodies of different subclasses. Data obtained using these particles were substantially in agreement with the results of the IgG-coated E experiments: IgG1, IgG2a, and IgG2b subclasses all bound significant amounts of ^{125}I -FcR, whereas only negligible binding was observed to either DNP-BSA beads or rabbit anti-DNP $F(ab')_2$ beads (Table II). In addition, no significant binding was observed to beads coated with an anti-DNP IgG3 monoclonal antibody. The binding of the ^{125}I -FcR to IgG2a- and IgG2b-coated beads was totally inhibited by both 2.4G2 IgG and Fab. This suggests that IgG2a and IgG2b may interact with the same or sterically adjacent sites on the purified receptor.

Although measurable binding was seen at extreme dilutions of ^{125}I -FcR ($<1 \times 10^{-3}$ U/ml), no meaningful association constant could be measured because the receptor was found to exist in large, presumably multivalent aggregates in PBS (Fig. 6). NP40 (0.5%) inhibited binding 80% relative to control, and 0.5% deoxycholate totally abolished activity (Table II).

(c) *Acid Lability of FcR Activity.* We examined the pH dependence of the binding reaction. Interestingly, ^{125}I -FcR binding fell off quite rapidly between pH 4 and 5 (Fig. 8), an acidity that approximates the intralysosomal environment (34). This inactivation of ^{125}I -FcR activity was reversible: upon neutralization of the reaction mixture, >80% of the initial binding activity was recovered after a brief (30 min) exposure to pH 4 buffer at room temperature.

TABLE II
Binding of ^{125}I -FcR to IgG-coated Particles

Experimental particle	Preincubation with	Binding (per- cent of input ^{125}I -FcR)
I. E		0.43
E IgG1		18
E IgG2a		28
E IgG2b		49
II. DNP-Sephadex		0.66
DNP-Sephadex-rabbit anti-DNP (ab') ₂		0.21
DNP-Sephadex-rabbit anti-DNP IgG		29
Rabbit IgG-Sephadex*		29
DNP-Sephadex-anti DNP IgG1		7.6
DNP-Sephadex-anti DNP IgG2b		28
DNP-Sephadex-anti DNP IgG2b	2.4G2 IgG	0.51
DNP-Sephadex-anti DNP IgG2b	2.4G2 Fab	0.18
IgG2a-Sephadex*		25
IgG2a-Sephadex*	2.4G2 IgG	0.58
IgG2a-Sephadex*	2.4G2 Fab	0.62
DNP-Sephadex-anti-DNP IgG3		0.41
III. DNP-Sephadex-rabbit anti-DNP IgG	—	35
DNP-Sephadex-rabbit anti-DNP IgG	0.5% NP40	8.0
DNP-Sephadex-rabbit anti-DNP IgG	0.5% deoxycholate	0.38
DNP-Sephadex-rabbit anti-DNP F(ab') ₂	—	0.21
DNP-Sephadex-rabbit anti-DNP F(ab') ₂	0.5% NP40	1.0
DNP-Sephadex-rabbit anti-DNP F(ab') ₂	0.5% deoxycholate	0.38

Erythrocytes (0.5 ml; 5% vol:vol) were incubated with U88 (IgG2a), SP/2 (IgG2b), or SP/3 (IgG1) ascites fluid (20 μl) at 4°C for 1 h, washed twice by centrifugation, and resuspended at 5% vol:vol in PBS that contained 2% FBS. ^{125}I -FcR from the Sephadex G-150 void-volume peak (20 μl ; 3×10^{-3} U; 40,000 cpm), adjusted with water to 0.154 M NaCl, was added to 50 μl of the erythrocyte suspension. Experiments II and III were performed with material from a Sephadex G-150 column (50 μl ; 1×10^{-3} U; 3,000 cpm). DNP-Sephadex was coated with 200 μg rabbit IgG or F(ab')₂/ml packed volume of beads or with 160 μl ascites fluid/ml packed volume. Preincubations of ^{125}I -FcR with 2.4G2 IgG or Fab were performed at a final concentration of 100 $\mu\text{g}/\text{ml}$ for 15 min at room temperature before the coated Sephadex beads were added. In experiment III, detergent was present in incubation and wash buffers.

* Antibody coupled to CNBr-activated Sephadex (Materials and Methods).

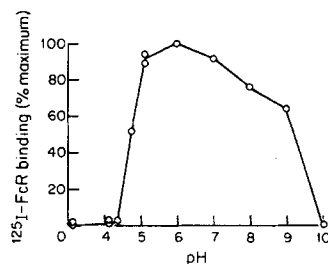


FIG. 8. Binding of ^{125}I -FcR as a function of pH. Binding of ^{125}I -FcR to DNP₁₂BSA-Sephadex beads coated with rabbit anti-DNP IgG or its F(ab')₂ fragment at different pH values was determined as described in Materials and Methods. The binding of ^{125}I -FcR to F(ab')₂-coated beads at pH 6 was 1.5% of the input; maximum binding to IgG-coated beads was 40%. Data presented have been corrected for binding to F(ab')₂-coated beads incubated in parallel at each pH.

Association of FcR with Membranes. We were interested in defining the nature of the association between the antigen recognized by 2.4G2 and the plasma membrane. By employing the assay for soluble FcR described above, we found that the receptor was not released from a preparation of total J774 membranes by sonication; 1.5 M NaCl; Na₂CO₃, pH 10.8; or 1 mM EDTA (Table III). Solubilization occurred only after the membranes were disrupted by nonionic detergent (0.5% NP40). Thus, the FcR can be defined as an integral membrane protein (35) that presumably extends into or across the lipid bilayer.

Identity of the Functionally Active FcR and 2.4G2 Antigen. Because the ¹²⁵I-FcR bound with high avidity to IgG-coated Sephadex, we could compare the proteins absorbed to the IgG-coated beads and the proteins immunoprecipitated by 2.4G2 Fab coupled to CNBr-activated Sephadex. The results (Fig. 9) show a virtual identity between the ¹²⁵I-protein that was immunoprecipitated by 2.4G2 Fab and the ¹²⁵I-protein that was bound by the IgG-coated Sephadex. The initial ¹²⁵I-FcR preparation contained some low *M_r* peptides (Fig. 9, left panel). Because these were absent in the re-isolated FcR (Fig. 9, middle and right panels) we conclude they have no relation to either antigenic or functional aspects of the FcR.

Binding of ¹²⁵I-proteins to FcR of Macrophages. To confirm previous experiments (9) that demonstrated that 2.4G2 Fab distinguished between the binding sites for IgG2b and IgG2a in the plasma membrane, we performed a series of quantitative inhibition of binding studies using the macrophage cell lines J774 and RAW 309 Cr.1. The binding of iodinated rabbit IgG in immune complexes (which binds primarily to the trypsin-resistant IgG2b receptor [3, 7, 8]) and that of ¹²⁵I-LPC-1 (an IgG2a myeloma protein that binds to a trypsin-sensitive site [6-8]) was determined in the presence and absence of 50 μg/ml 2.4G2 Fab (Fig. 10). The number of sites for IgG2a at saturation was decreased by only 15% in the presence of 50 μg/ml of 2.4G2 Fab; the

TABLE III
Solubilization of the 2.4G2 Antigen from J774 Membranes

Treatment	Antigen solubilized
	<i>U</i>
I. 0.5% NP40 1 mM PMSF 0.2 U/ml aprotinin	29.15
II. 1.5 M NaCl	6.85
III. 0.05 M Na ₂ CO ₃ , pH 10.8	5.70
IV. 25 mM Hepes, pH 7.6 1 mM PMSF 0.2 U/ml aprotinin 1 mM EDTA	6.80

Crude J774 membranes were prepared as described in Materials and Methods. Equal amounts of membranes were then resuspended in the indicated solutions and incubated with constant agitation for 10 min at room temperature in a final vol of 0.5 ml. Samples were then centrifuged for 1 h at 100,000 *g* (4°C) and the resulting supernates assayed for soluble FcR (Materials and Methods). 0.5% NP40 was found to solubilize ~60% of the detectable antigenic activity from J774 membrane preparations.

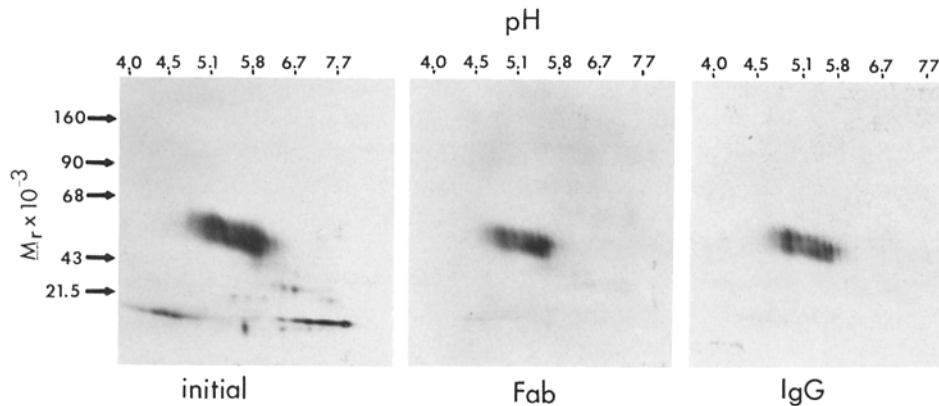


FIG. 9. Analysis of iodinated proteins that bind to 2.4G2 Fab-Sephadex and rabbit IgG-Sephadex. Left panel, ^{125}I -FcR preparation analyzed by two-dimensional gel electrophoresis with the isoelectric focusing (horizontal direction) performed under equilibrium conditions. Center, ^{125}I -proteins bound by 2.4G2 Fab coupled to CNBr-activated Sephadex, eluted with 1% SDS, and analyzed as above. Right, ^{125}I -proteins bound by DNP₁₂BSA Sephadex coated with rabbit anti-DNP IgG. See Materials and Methods for details.

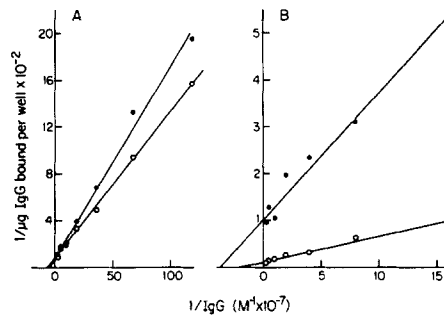


FIG. 10. Binding of IgG2a and soluble rabbit immune complexes to RAW 309 Cr.1 cells in the presence and absence of 50 $\mu\text{g}/\text{ml}$ of 2.4G2 Fab. RAW 309 Cr.1 cells (5×10^5) seeded in 16-mm wells were incubated at 4°C for 60 min with a constant input of ^{125}I -LPC-1 (10 ng; 6.2×10^7 cpm/ μg) and unlabeled LPC-1 in a final vol of 0.3 ml (A); or with ^{125}I -rabbit anti-DNP IgG DNP₁₂BSA complexes (1.48×10^4 cpm/ μg) formed at an antibody:antigen ratio of 5:1 (B). Background (nonsaturable) binding was assessed with low specific activity LPC-1 mixtures and with F(ab)₂ complexes. (○) Binding in the absence of 2.4G2 Fab; (●) binding in the presence of 50 $\mu\text{g}/\text{ml}$ of 2.4G2 Fab.

association constant (K_a) for LPC-1 was unaffected (Table IV). In contrast, addition of the 2.4G2 fragment resulted in a dramatic (10-fold) inhibition of the binding of rabbit IgG in immune complexes, to approximately the same number of sites (80,000) as IgG2a. This may be significant because rabbit immune complexes bind to both trypsin-sensitive and -resistant sites (3, 8). Thus, in agreement with previous results in which the binding of IgG2a-coated E to J774 was examined, 2.4G2 Fab had little effect on the binding of monomeric IgG2a to RAW 309 Cr.1. Similarly, 2.4G2 Fab had little effect on the binding of IgG2a to J774 cells (data not shown). There were more binding sites at saturation for rabbit IgG in immune complexes (750,000) than for IgG2a (107,000) or 2.4G2 Fab (350,000), which also argues that the FcR for IgG2a and rabbit IgG in complexes differ.

The lack of inhibition observed by 2.4G2 Fab for IgG2a binding could be explained

TABLE IV
Binding of Immunoglobulins and 2.4G2 Fab to Macrophage Cell Lines

Cell	¹²⁵ I-protein tested	Addition	K _a	Sites/cell	k _{off}	k _{on}
			M ⁻¹		min ⁻¹	(M ⁻¹ ·min ⁻¹)
RAW 309 Cr.1	LPC-1 (IgG2a, κ)	—	6.0 × 10 ⁷	107,000		
	LPC-1 (IgG2a, κ)	2.4G2 Fab (50 μg/ml)	5.4 × 10 ⁷	91,000		
	Rabbit anti-DNP IgG	—	1.9 × 10 ⁷	750,000		
	DNP ₁₂ BSA complexes*					
	Rabbit anti-DNP IgG	2.4G2 Fab (50 μg/ml)	3.6 × 10 ⁷	80,000		
	DNP ₁₂ BSA complexes*					
	2.4G2 Fab	—	1.7 × 10 ⁸	350,000		
J774	2.4G2 Fab	—	1.3 × 10 ⁸ ‡		2.3 × 10 ⁻³	3 × 10 ⁶
P388D ₁	LPC-1 (IgG2a, κ)	—	1.1 × 10 ⁸ (6)		8.3 × 10 ⁻² (6)	9 × 10 ⁶ (6)

All assays were performed at 4°C essentially as described in Materials and Methods. Pseudo-first-order K_a were determined for concentrations of Fab from 2 × 10⁻⁸ M to 8 × 10⁻⁸ M. The slope of these constants plotted against concentration was used to determine k_{on} (36).

* The rabbit IgG was labeled. Binding and saturation data are expressed with regard to IgG concentration, not complex concentration.

‡ From k_{on}/k_{off}.

if the K_a of IgG2a were much greater than that of 2.4G2 Fab. However the K_a of 2.4G2 Fab at 4°C was >1 × 10⁹ M⁻¹ and the t_{1/2} for dissociation >4 h; both of these values are far greater than the corresponding values determined previously for IgG2a binding: ~1 × 10⁸ M⁻¹ and 8 min (6). The inhibition studies 2.4G2 Fab on intact macrophages, the binding of the purified FcR to both immobilized IgG2a and IgG2b, and the inhibition of the purified FcR binding to IgG2a and IgG2b by 2.4G2 Fab all indicate that the purified FcR has a somewhat altered specificity from the molecule in the macrophage membrane.

Discussion

The use of cell hybridization to raise monospecific, homogeneous antibodies as pioneered by Köhler and Milstein (37) can greatly facilitate the study of the biochemistry and function of cell surface receptors. Recently, we reported (9) the isolation of a rat monoclonal hybrid, 2.4G2, that secretes an IgG directed against the trypsin-resistant mouse FcR, which binds aggregated IgG1 and IgG2b. In the present study, we have used this antibody as an affinity reagent to isolate FcR in quantities sufficient for biochemical analysis. The high degree of specificity and affinity of 2.4G2 Fab for its antigen enabled the purification of the receptor to apparent homogeneity in one step. The mouse macrophage cell line J774 was chosen as the source of FcR because quantitative binding studies using ¹²⁵I-2.4G2 Fab (9) indicated that these cells possessed the greatest number of receptor sites/cell (>500,000) of the cell lines tested. Furthermore, J774 cells could be readily grown as subcutaneous tumors in syngeneic mice, thus providing a convenient means to prepare large amounts of tissue.

FcR isolated from J774 cell lysates using 2.4G2 Fab-Sepharose was found to consist of two components with 60,000 and 47,000 M_r. That these polypeptides were glycosylated was indicated by labeling with the galactose oxidase-NaB[³H]₄ procedure (22) and by their affinity for Con A-Sepharose. Similarly, the presence of sialic acid was demonstrated by the shift to a more basic isoelectric point after digestion with neuraminidase. However, the most significant aspect of this work was the demonstration that the isolated antigen exhibited FcR activity in vitro.

Two different methods were used to demonstrate functional activity. First, the

purified receptor was found to dramatically enhance the hemagglutination of E sensitized with rabbit antibody, mouse IgG2b, or IgG2a. Rabbit IgG-coated E were agglutinated in the presence of even small quantities of FcR (0.1 U/ml), forming tight, nondispersable clumps. The enhancement was specific for the Fc domain of IgG, because E_{TNP} coated with an equivalent hemagglutinating concentration of rabbit anti-DNP F(ab')₂ were unaffected by even high concentrations (5 U/ml) of FcR.

To provide a more readily quantifiable measure of activity, purified FcR was iodinated *in vitro* to allow us to measure the binding of the FcR to IgG-coated surfaces. These experiments demonstrated that up to 50% of the iodinated protein could bind to IgG. Furthermore, this interaction, like the hemagglutination enhancement noted above, was dependent on the Fc domain, because the binding to nonopsonized or F(ab')₂-coated beads was <1%. Because the iodinated FcR bound with high avidity to IgG-coated beads, we could demonstrate directly that the antigen immunoprecipitated by 2.4G2 Fab and immune complexes was the same, thus proving that the antigen recognized by 2.4G2 is indeed an FcR.

Interestingly, however, the purified FcR did not exhibit the same IgG subclass specificity in solution as in the plasma membrane of intact cells. Considerable evidence (2-4) suggests that IgG2a and IgG2b bind to different sites based on the lack of inhibition of each subclass for binding of the other. Indeed, as discussed previously, the 2.4G2 Fab inhibited the binding to macrophages of IgG1 and IgG2b, but not IgG2a immune aggregates. Of the mouse IgG subclasses, the ¹²⁵I-FcR failed to bind only to IgG3 immune aggregates. Furthermore, the binding of ¹²⁵I-FcR to both IgG2a- and IgG2b-coated beads was totally inhibited by 2.4G2 Fab. This is quite different from the inhibition of IgG2a binding to intact cells, which is inhibited only 15% by inclusion of 1×10^{-6} M Fab in the incubation mixtures. It is unlikely that this lack of inhibition is a result greater affinity of IgG2a for the FcR than 2.4G2 Fab because the K_a for 2.4G2 Fab is 10^9 M⁻¹, 10-fold higher than that of IgG2a.

Any of several factors might account for the loss of specificity observed for the purified FcR. For example, removal of the FcR from the lipid bilayer or from other accessory component(s) (e.g., specific phospholipids, glycolipids, or other membrane proteins) in the plasma membrane could cause a conformational change in the active site of the FcR. Similarly, the tendency of the receptor to form aggregates or protein micelles in aqueous solution, behavior previously reported for other integral membrane proteins (38), might result in altered affinities for all IgG subclasses. For both acetylcholine receptor (39), and IgE FcR (40), the solubilized proteins show greatly increased affinity and somewhat altered specificity relative to the membrane-associated molecule. Alternatively, it is possible that both the IgG2b- and the IgG2a-binding sites are present on the same molecule. In this instance, the 2.4G2 antigenic determinant would have to be close to the IgG2b site, with the 15% inhibition of IgG2a binding by 2.4G2 Fab consistent with a small degree of steric hinderance. However, it is clear from the quantitative binding data on intact cells that there are many more binding sites for the 2.4G2 Fab than for IgG2a on both thioglycollate-elicited macrophages and the RAW 309 Cr.1 line. Therefore, any proposal that the 2.4G2 antigen is a component of the trypsin-sensitive IgG2a FcR will have to account for that disparity. Possibilities under consideration are: (a) the IgG2a FcR site is formed by aggregation of FcR, or (b) is formed by association of FcR with other

membrane protein(s) present in smaller amounts, and that these associations are abolished (or the accessory proteins degraded) when the cells are treated with trypsin.

In this regard, it is interesting to note that Anderson and Grey (41) have reported that crude lysates of the P388D₁ macrophage line from which detergent was removed by SM-2 BioBeads had two activities separable by velocity sedimentation. Material capable of binding IgG aggregates (but not IgG2a) was found to sediment at >19S, whereas material sedimenting at 4–5S could bind IgG2a. The IgG aggregate activity was phospholipase C sensitive, in contrast to the present results with a highly purified FcR preparation. They also observed that the T cell line S49.1, which we found bears the 2.4G2 antigen, did not have IgG2a binding activity. We are at present isolating the S49.1 receptor to examine its subclass specificity.

2.4G2 Fab was also employed to investigate the structure of the FcR expressed by several other macrophage and lymphoid cell lines and cell types. As shown in Fig. 1, a significant degree of heterogeneity was observed. After SDS-PAGE, diffuse radioactive bands were found that ranged from 47,000 M_r (thioglycollate-elicited peritoneal macrophages) to >70,000 M_r (the B lymphocyte cell line WEHI-231). Each of these molecules bears the common 2.4G2 antigenic determinant, and, moreover, the FcR activity of each cell type was blocked by prior incubation with 2.4G2 (9). Although we do not know the reason for these differences in M_r of FcR, there is some precedent for such heterogeneity of antigens isolated from different cell types by use of a monoclonal antibody. For example the rat leukocyte common antigen recognized by MRC OX-1 immunoprecipitates polypeptides of 150,000 M_r from thymocytes, 170,000 M_r from T lymphocytes, 200,000 M_r from B lymphocytes, and 130,000–150,000 from bone marrow cells (42).

One possible source of the observed heterogeneity in M_r of FcR isolated from different sources could be differences in glycosylation. Alternatively, such heterogeneity might reflect differing levels of proteases secreted by the cells. Trypsin digestion of intact cells does not inactivate FcR activity for IgG2b or rabbit immune complexes (7), nor does it destroy the 2.4G2 antigenic site. However, trypsinization did decrease the M_r of the J774 FcR (Fig. 2) apparently by converting the 60,000- M_r polypeptide to one similar in size to the smaller 47,000- M_r component. Consistent with the possibility that FcR may be self-digested in this way, is that thioglycollate-elicited macrophages, which release neutral proteases (43), had the lowest M_r FcR of all cell types examined. Whatever the source of the observed size heterogeneity among different FcR, it is nonetheless interesting that a monoclonal anti-FcR antibody originally raised against the receptor expressed by the macrophage cell lines J774 and P388D₁ can recognize FcR expressed by cells of greatly divergent lineages.

Although previous estimates of FcR M_r have spanned a wide range (<20,000–255,000), there have been several reports which agree fairly well with our results. Loube et al. (10) isolated a 57,000- M_r protein of broad electrophoretic mobility from the P388D₁ macrophage line; Cunningham-Rundles et al. (11) reported a trypsin-resistant FcR from human peripheral mononuclear cells of 60,000 M_r ; Kulczycki et al. (12) reported isolation of a receptor from rabbit alveolar macrophages of ~50,000 M_r .

However, the approach we have employed to isolate FcR is substantially different from such previous attempts. Earlier efforts have relied on affinity chromatography of lysates over IgG coupled to various supports. Such procedures did not provide for

the estimation of yields or recoveries, and the only control possible for specificity was to compare species recovered from control adsorbents coated with Fab or F(ab')₂ fragments. In addition, in most cases, only trace amounts of material were isolated, labeled isotopically with either ¹²⁵I after cell surface iodination, or labeled biosynthetically after incorporation of oligosaccharide precursors. Hence, contamination by cytoplasmic proteins such as actin (which has considerable affinity for IgG [44]) could not be assessed.

The only previous study in which activity of the isolated material was reported by Kulczycki et al. (12), who isolated ¹²⁵I- or [¹⁴C]glucosamine-labeled molecules from NP40 lysates of rabbit alveolar macrophages. After elution from IgG-Sepharose columns with 0.5 M acetic acid, the adsorbed material had a broad ~50,000 *M_r* and was capable of rebinding to IgG-Sepharose, a result that is agreement with our results. It should be emphasized that we have relied solely on the antigenicity of the FcR recognized by the anti-FcR antibody, to purify the receptor from cell lysates, and that the capability of the isolated protein to bind to IgG in an acid-labile fashion thus represented a completely independent property of the molecule. In addition, our preliminary results indicate that a rabbit antiserum prepared against the isolated antigen also specifically inhibits FcR activity of mouse macrophages and immunoprecipitates identical polypeptides.

The availability of pure Fc receptor will make possible structural studies of functional domains of the receptor after proteolytic cleavage, and reconstitution experiments to study the effect of insertion into a lipid bilayer on subclass specificity. Comparison of the detailed structure of this FcR with the IgE FcR, which seems quite comparable in its acid lability and *M_r* (45), may demonstrate the presence of a family of receptors with closely related structures but with exquisitely defined specificities.

Summary

We recently reported the isolation of a rat monoclonal antibody designated 2.4G2 (9) that is directed against the mouse trypsin-resistant Fc receptor (FcR) for IgG2b and IgG1 immune aggregates. We have now utilized the Fab fragment of 2.4G2 as an affinity reagent to purify FcR from the macrophage cell line J774 to apparent homogeneity. The antigen isolated from J774 cells consisted of two general types of polypeptides with broad electrophoretic mobilities of ~60,000 and 47,000 mol wt. Similar broad bands ranging from 47,000 to 70,000 mol wt were isolated from various FcR-bearing cell lines of B, T, and null lymphocyte, as well as of macrophage origin. J774 FcR was judged to be a glycoprotein based on the sensitivity of its isoelectric point to neuraminidase digestion, its labeling with galactose oxidase/NaB[³H₄], and its binding to concanavalin A-Sepharose. In phosphate-buffered saline, the isolated protein formed large aggregates that were shown to retain FcR activity, albeit with a somewhat altered IgG subclass specificity. The FcR agglutinated erythrocytes that were coated with both IgG2b and IgG2a that did not otherwise hemagglutinate. In addition, iodinated FcR bound to Sephadex beads coated with rabbit IgG, mouse IgG1, IgG2b, and IgG2a, but not to beads coated with mouse IgG3 or rabbit F(ab')₂ fragments. The binding of the purified receptor to all IgG classes was inhibited by the Fab fragments of 2.4G2. In contrast, the binding of IgG2a to intact macrophages was

inhibited by 2.4G2 Fab by only 15%, whereas rabbit IgG immune aggregate binding was almost completely abolished.

We would like to thank Marie McGettigan and Helen Plutner for their expert and tireless assistance; Gianni Piperno for his advice and help in the use of two-dimensional gel electrophoresis; Carl Nathan, Sam Silverstein, and Victoria Freedman for their editorial suggestions; Judy Adams for her assistance with photographic work; and Marie Grossi for help in preparing the manuscript. We also thank Professor Zanvil A. Cohn for his continued advice and support.

Received for publication 16 June 1980.

References

1. Dickler, H. B. 1976. Lymphocyte receptors for immunoglobulin. *Adv. Immunol.* **24**:167.
2. Heusser, C. H., C. L. Anderson, and H. M. Grey. 1977. Receptors for IgG: subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. *J. Exp. Med.* **145**:1316.
3. Diamond, B., B. R. Bloom, and M. D. Scharff. 1978. The Fc receptors of primary and cultured phagocytic cells studied with homogeneous antibodies. *J. Immunol.* **121**:1329.
4. Walker, W. S. 1976. Separate Fc-receptors for immunoglobulins IgG2a and IgG2b on an established cell line of mouse macrophages. *J. Immunol.* **116**:911.
5. Anderson, C. L., and H. M. Grey. 1974. Receptors for aggregated IgG on mouse lymphocytes. Their presence on thymocytes, thymus-derived, and bone marrow-derived lymphocytes. *J. Exp. Med.* **139**:1175.
6. Unkeless, J. C., and H. N. Eisen. 1975. Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. *J. Exp. Med.* **142**:1520.
7. Unkeless, J. C. 1977. The presence of two Fc receptors on mouse macrophages: evidence from a variant cell line and differential trypsin sensitivity. *J. Exp. Med.* **145**:931.
8. Unkeless, J. C., G. Kaplan, H. Plutner, and Z. A. Cohn. 1979. Fc-receptor variants of a mouse macrophage cell line. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1400.
9. Unkeless, J. C. 1979. Characterization of monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* **150**:580.
10. Loube, S. R., T. C. McNabb, and K. J. Dorrington. 1978. Isolation of an Fc_γ-binding protein from the cell membrane of a macrophage-like cell line (P388D₁) after detergent solubilization. *J. Immunol.* **120**:709.
11. Cunningham-Rundles, C., F. P. Siegal, and R. A. Good. 1978. Isolation and characterization of a human mononuclear cell Fc receptor. *Immunochemistry.* **15**:365.
12. Kulczycki, A., Jr., V. Krause, C. C. Killian, and J. P. Atkinson. 1980. Purification of Fc_γ receptor from rabbit alveolar macrophages that retains ligand-binding activity. *J. Immunol.* **124**:2772.
13. Rask, L., L. Klareskog, L. Östberg, and P. A. Peterson. 1975. Isolation and properties of a murine spleen cell Fc receptor. *Nature (Lond.)* **257**:231.
14. D'Urso-Coward, M., and R. E. Cone. 1978. Membrane proteins of the P388D₁ macrophage cell line: isolation of membrane polypeptides that bind to the Fc portion of aggregated IgG. *J. Immunol.* **121**:1973.
15. Bourgois, A., E. R. Abney, and R. M. E. Parkhouse. 1977. Structure of mouse Fc receptor. *Eur. J. Immunol.* **7**:691.
16. Sire, J., B. Kahn-Perles, A. Collé, and A. Bourgois. 1980. Biochemical characterization of an Fc receptor of rabbit lymphocytes. *Eur. J. Immunol.* **10**:116.
17. Cooper, S. M., Y. Sambray, and G. J. Friou. 1977. Isolation of separate Fc receptors for IgG complexed to antigen and native IgG from a murine leukemia. *Nature (Lond.)* **270**:253.
18. Cheng, C. M., and J. Hawiger. 1979. Affinity isolation and characterization of immuno-

- globulin G Fc fragment-binding glycoprotein from human platelets. *J. Biol. Chem.* **254**:2165.
19. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **121**:153.
 20. Hubbard, A. L., and Z. A. Cohn. 1975. Externally disposed plasma membrane proteins. I. Enzymatic iodination of mouse L cells. *J. Cell Biol.* **64**:438.
 21. Mellman, I. S., R. M. Steinman, J. C. Unkeless, and Z. A. Cohn. 1980. Selective iodination and polypeptide composition of pinocytotic vesicles. *J. Cell Biol.* **86**:712.
 22. Critchley, D. R. 1974. Cell surface proteins of NILI hamster fibroblasts labeled by a galactose oxidase, tritiated borohydride method. *Cell.* **3**:121.
 23. Sonoda, S., and M. Schlamowitz. 1970. Studies of ¹²⁵I trace labeling of immunoglobulin G by chloramine T. *Immunochemistry.* **7**:885.
 24. Kaplan, G., J. C. Unkeless, and Z. A. Cohn. 1979. Insertion and turnover of macrophage plasma membrane proteins. *Proc. Natl. Acad. Sci. U. S. A.* **76**:3824.
 25. Neville, D. M., Jr., and H. Glassmann. 1974. Molecular weight determination of membrane protein and glycoprotein subunits by discontinuous gel electrophoresis in dodecyl sulfate. *Methods Enzymol.* **32**:92.
 26. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83.
 27. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* **12**:1133.
 28. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007.
 29. March, S. C., I. Barikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* **60**:149.
 30. McMaster, R. W., and A. F. Williams. 1979. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol.* **9**:426.
 31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1971. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
 32. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248.
 33. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenyl plaque assay. Primary responses of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132**:575.
 34. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3327.
 35. Singer, S. J. 1974. The molecular organization of membranes. *Annu. Rev. Biochem.* **43**:805.
 36. Schonbrunn, A., and A. H. Tashjian, Jr. 1978. Characterization of functional receptors for somatostatin in rat pituitary cells in culture. *J. Biol. Chem.* **253**:6473.
 37. Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.)*. **256**:495.
 38. Tanford, C., and J. A. Reynolds. 1976. Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta.* **457**:133.
 39. Simons, K., A. Helenius, K. Leonard, M. Sarvaś, and M. J. Gething. 1978. Formation of protein micelles from amphiphilic membrane proteins. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5306.
 40. Rossi, G., S. A. Newman, and H. Metzger. 1977. Assay and partial characterization of the solubilized cell surface receptor for immunoglobulin E. *J. Biol. Chem.* **252**:704.
 41. Anderson, C. L., and H. M. Grey. 1978. Physicochemical separation of two distinct Fc receptors on murine macrophage-like cell lines. *J. Immunol.* **121**:648.

42. Standring, R., W. R. McMaster, C. A. Sunderland, and A. F. Williams. 1978. The predominant heavily glycosylated glycoproteins at the surface of rat lymphoid cells are differentiation antigens. *Eur. J. Immunol.* **8**:832.
43. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139**:834.
44. Flanagan, J., and G. L. E. Koch. 1978. Cross-linked surface Ig attaches to actin. *Nature (Lond.)*. **273**:278.
45. Kulczycki, A., Jr., B. L. Hempstead, S. L. Hofmann, E. W. Wood, and C. W. Parker. 1979. The cell surface receptor for immunoglobulin E. II. Properties of the purified biologically active receptor. *J. Biol. Chem.* **254**:3194.