THE PRESENCE OF TWO Fc RECEPTORS ON MOUSE MACROPHAGES: EVIDENCE FROM A VARIANT CELL LINE AND DIFFERENTIAL TRYPSIN SENSITIVITY*

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Fc receptors are found on a wide variety of cells including macrophages (1–9), polymorphonuclear leukocytes (PMN)[1] (10), B cells (11, 12), some classes of T cells (13), mast cells (14), and herpes virus-infected cells (15). The role the receptors play in the physiology of these cells has not been defined in all cases; however, for the macrophage and PMN these receptors function, at least in part, in the recognition and ingestion of immune complexes.

Fc receptors of macrophages from rabbit (3), guinea pig (1), and man (4) are resistant to trypsin treatment. The mouse macrophage Fc receptor that binds rabbit antibody-antigen complexes is also resistant to trypsinization (5). However, other studies (16–18) on the binding to mouse macrophages of "early" mouse cytophilic antibody show that these antibodies bind to a trypsin-sensitive receptor, whereas "late" or hyperimmune antibody binds to a trypsin-resistant site. This suggests that there are two classes of Fc receptors on mouse macrophages. Askenase and Hayden (18) found, based on inhibition experiments with IgG2a-specific antisera, that the class of mouse IgG which binds to the trypsin-sensitive receptor is IgG2a. In previous work (8) we characterized the affinity of binding of monomeric mouse myeloma proteins to mouse macrophages and a macrophage cell line, $P388D_1$, and demonstrated that the Fc receptor for monomeric mouse IgG2a was sensitive to trypsinization, and could be regenerated by the macrophage in the absence of serum.

In this paper I show that the mouse macrophage line, $P388D_1$, has two Fc receptors, one which binds mouse IgG2a, and another which binds rabbit IgG in antigen-antibody complexes. Genetic and biochemical evidence for separate receptors is presented: (*a*) A variant line which is unaltered in its ability to bind IgG2a but differs markedly in binding of rabbit antibody-antigen com-

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; C′, complement; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HIFCS, heat-inactivated fetal calf serum; ^{125}IUDR, ^{125}I-iododeoxyuridine; KLH, keyhold limpet hemocyanin; MEM, alpha-modified Eagle's minimum essential medium; PBS, phosphate buffered saline; PMN, polymorphonuclear leukocyte; TNP, trinitrophenyl.

plexes was isolated from a clone of the P388D$_1$ line. (*b*) The receptors for IgG2a and antigen-antibody complexes differ in sensitivity to treatment with trypsin. Furthermore, the trypsin-resistant receptor that binds rabbit antigen-antibody complexes does not seem to bind uncomplexed rabbit IgG.

Materials and Methods

Cell Culture. The P388D$_1$ line (6, 19) was grown as described previously (8). Adherent macrophages were transferred using the local anesthetic lidocaine as described by Rabinovitch and DeStephano (20). Cells were incubated with 12 mM lidocaine in alpha-modified Eagle's minimum essential medium (MEM) with 10% heat-inactivated fetal calf serum (HIFCS) for 10–15 min, after which they were washed gently off the dish with a pipette, resuspended in medium, and replated.

The P388D$_1$ line was cloned by seeding cells in 96-well flat bottom trays at an average density of one cell per well. A 1:3 ratio of medium conditioned by P388D$_1$ cells growing in spinner culture to fresh medium was used; the cloning efficiency under these conditions was 50%. The trays were incubated long enough (2 wk) to allow macroscopic colonies to form, after which they were recloned. Clones were then expended and frozen in liquid nitrogen in MEM containing 10% HIFCS and 10% (vol/vol) glycerol. Mouse peritoneal macrophages were harvested from C57BL/6 ♀, 20–25 g in weight, 4 days after intraperitoneal injection of 0.75 ml of thioglycollate medium, as described previously (21).

Myeloma Proteins. The following myeloma proteins of different subclasses were used: MOPC-21, IgG1; LPC-1 and UPC-10, IgG2a; and MPC-11, IgG2b. All myeloma proteins used have κ-light chains and react with rabbit anti-κ antiserum. The corresponding tumors were obtained from Dr. Michael Potter, National Cancer Institute, Bethesda, Md., or from Litton Bionetics, Inc., Silver Spring, Md., and were passaged as described previously (8). Myeloma proteins were purified by ion exchange chromatography and iodinated using chloramine-T as described previously (8, 22).

Selection of Variants. Variant clones were selected using a complement (C′)-dependent selection protocol. Cloned P388D$_1$ cells (5×10^6 cells/100-mm dish) were incubated at room temperature for 15 min with a 1:1 mixture of phosphate-buffered saline containing 1 mg/ml of bovine serum albumin (PBS-BSA; 0.137 M NaCl, 3mM KCl, 16 mM Na_2HPO_4, and 2 mM KH_2PO_4) and L-15 medium, and a final concentration of 15 μg/ml of LPC-1 mouse myeloma protein. After addition of rabbit anti-κ (10 μg/ml) and rabbit C′ (20% vol/vol), the dishes were placed at 37°C for 45 min, washed once with Hanks' balanced salt solution (HBSS), and refed with medium. The titer of the rabbit anti-κ IgG, determined by quantitative precipitin curve, was 1 mg/ml. Rabbit C′ was sterilized by ultrafiltration through a 0.45 μm Millipore filter; antibody was sterilized by UV irradiation. The selection protocol was repeated on successive days, the cells were allowed to grow for 1–2 wk, and the selection protocol was repeated as before. Surviving colonies were inspected and colonies showing minimal cytotoxicity were isolated using 4-mm stainless steel cloning wells. Lines thus isolated were then cloned.

Preparation of Dinitrophenyl (DNP)- and Trinitrophenyl (TNP)-Substituted Proteins. TNP- and DNP-substituted proteins were prepared by reacting the protein dissolved in 4% potassium carbonate with picryl sulfonic acid or 2-4 dinitrobenzene sulfonic acid, except when a low degree of DNP substitution was desired, in which case 0.25% K_2CO_3 buffer (pH 10.2) was used. The extent of substitution was calculated from the molar extinction coefficients of ϵ-TNP-L-lysine at 348 nm (15,400) and ϵ-DNP-L-lysine at 360 nm (17,530) (23). The concentration of substituted DNP-BSA was calculated as follows: BSA(mg/ml) = (A280nm − 0.33 A360nm)/(0.7).

Isolation of Rabbit Anti-DNP Antibody. The method used is a modification of a method developed by Eisen et al. (24). A TNP-BSA immunoabsorbant was prepared by coupling 5 mg of TNP_{16}BSA/g of Sepharose 4-B using CNBr activation as described by Cuatrecasas (25). Hyperimmune serum (20 ml) from a rabbit repeatedly immunized intradermally with DNP-keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant was diluted 1:2 with 0.1 M Tris-HCl, pH 7.8, and passed over a 10 ml column of TNP_{16}BSA-Sepharose 4-B. The column was washed with 0.1 M Tris-HCl, and bound protein was then eluted with 0.1 M dinitrophenol in 0.1 Tris-HCl, pH 7.8. Most of the dinitrophenol was removed from pooled fractions by chromatography on Sephadex G-25 using 0.15 M NaCl, 0.01 M Tris-HCl, pH 8.1, as eluant. Remaining dinitrophenol (1.3 mol/mol IgG) was removed by passing the protein in the same Tris-saline buffer through a Dowex-1

column (Dowex-1 X-8, 100–200 mesh, Cl form, 2 ml bed; Dow Chemical Co., Midland, Mich.) at 56°C. The yield was 66 mg protein and the molar ratio of dinitrophenol:IgG was less than 0.1. The antibody was of high affinity; DNP-ε-amino caproate, a good ligand, bound so tightly that it could not be removed by dialysis, chromatography on Sephadex, or chromatography on Dowex-1 at 65°C.

Preparation of F(ab) and F(ab')$_2$ Fragments. The F(ab')$_2$ fragment was prepared from rabbit anti-DNP IgG by digestion with pepsin (26). The contamination of the F(ab')$_2$ by native IgG was judged to be less than 5% by electrophoresis of ^{125}I-F(ab')$_2$ in a 10% sodium dodecyl sulfate-polyacrylamide gel. Agglutination of TNP-sheep erythrocytes (SRBC) by the F(ab')$_2$ and native anti-DNP Ig gave end points of 20 μg/ml and 30 μg/ml, respectively. The F(ab) fragment from the myeloma protein LPC-1 was prepared by papain digestion as described previously (8).

Cytotoxicity Assays. Cytotoxicity was determined by the release of ^{125}I-iododeoxyuridine (^{125}IUDR) from labeled cells (27, 28). Cells (3×10^5) in 16-mm wells were labeled by exposure to 1–2 μCi of ^{125}IUDR in 0.5 ml of RPMI-1640 containing 10% HIFCS for 2 h at 37°C, washed, and incubated for 1–2 h in MEM before use in cytotoxicity assays. After the experiment the supernate was reserved and the cells were treated for 30 min at 37°C with 0.25% tissue culture trypsin (0.5 ml) to lyse damaged cells. The combined first supernate and the trypsin was then assayed for radioactivity. Radioactivity remaining in viable cells was assayed by wiping the wells with cotton swabs. The cytotoxicity was calculated as follows: cytotoxicity = (% experimental release − % spontaneous release)/(100 − % spontaneous release). All experiments were done in duplicate and agreed within 10%. Spontaneous release varied from 5 to 20%, but was consistent in any one experiment.

Binding Assays. Assays of the binding of LPC-1 were done as described (8), using a constant input of ^{125}I-LPC-1 and varying the amount of unlabeled LPC-1. Assays for binding of the rabbit anti-DNP DNP-BSA complexes differed in that increasing amounts of preformed radioactive complexes of constant specific activity were used. Cultures were incubated for 1 h at 4°C and for 30 min at 37°C, the supernate was removed, and the trays were dipped into PBS at 4°C to remove unbound complexes. IgG bound to the cells was released by treatment with tissue culture trypsin at 37°C for 30 min. The binding of the complexes to NIH 3T3 cells was used to estimate the nonspecific binding. In some experiments cells were trypsinized before binding studies with 1 mg/ml diphenyl carbamyl chloride-treated trypsin in PBS containing 0.1% glucose for 15 min at 37°C. After aspiration of the trypsin, cells were then washed in MEM containing 10% HIFCS before the binding assays were performed. All assays were in duplicate and variation from the mean usually did not exceed 10%.

Immunofluorescence. Goat anti-mouse IgG and goat anti-rabbit IgG were labeled with fluorescein isothiocyanate (FITC). The goat anti-rabbit IgG was first passed over a small IgG2a-Sepharose 4-B column to remove any cross-reacting anti-mouse IgG antibody. The 280 nm/495 nm absorption ratio of the conjugates was 3.0.

Cells seeded on 12-mm glass cover slips were incubated at 37°C for 45 min in a 1:1 mixture of PBS-BSA:L-15 containing either 15 μg/ml of LPC-1 or 15 μg/ml of LPC-1 and 10 μg/ml of rabbit anti-κ. The cover slips were washed in PBS and stained at 4°C for 1 h with 120 μg/ml of either FITC-goat anti-rabbit or FITC-goat anti-mouse IgG in 1:1 PBS-BSA:L-15. The cover slips were again washed, fixed at room temperature for 30 min in 3.5% formaldehyde in PBS, and mounted in PBS containing 30% (vol/vol) glycerol and 15% (wt/vol) Gelvatol 20/30. Specimens were viewed using a 63× oil immersion lens on a Zeiss microscope (PM II; Carl Zeiss, Inc., New York) equipped with epifluorescence optics. Photographs (30-s exposures) were taken on High Speed Ektachrome and processed by Kodak (Eastman Kodak Co., Rochester, N. Y.) to raise the ASA to 400. The slides were copied onto Super XX pan 4142 film using a green filter to reduce yellow-orange lysosomal autofluorescence.

Preparation of SRBC. Myeloma proteins were coupled to SRBC using glutaraldehyde (29). SRBC derivatized with TNP were prepared as described by Rittenberg and Pratt (30). To prepare IgG-coated TNP-SRBC, 5×10^8 TNP-SRBC were added to 10 ml of 1:1 mixture of PBS-BSA:L-15 containing 2.8 μg rabbit anti-DNP IgG, incubated at room temperature for 30 min, washed, and resuspended in PBS-BSA:L-15.

Preparation of Protein-Coated Sephadex G-25. Superfine Sephadex G-25 (10–40 μm diameter) was activated with CNBr (25), and 1 g portions (wet weight) of activated Sephadex were added to 4 ml of PBS containing 1 mg/ml of either LPC-1, rabbit anti-DNP IgG, DNP$_{10.9}$BSA, or BSA and

the mixture stirred overnight. The BSA- and DNP-BSA-coated beads were incubated at room temperature with 1 mg/ml of rabbit anti-DNP IgG, and then washed repeatedly by centrifugation. The amount of IgG bound per gram of Sephadex was determined from the specific activity of the IgG used in the initial incubations and was as follows: LPC-1 Sephadex, 900 μg; rabbit anti-DNP-Sephadex, 440 μg: $DNP_{10.9}$BSA-Sephadex incubated with anti-DNP IgG, 180 μg; and BSA-Sephadex incubated with anti-DNP IgG, 3 μg.

Reagents were obtained from the following sources: MEM (K. C. Biological Inc., Lenexa, Kans.); penicillin, streptomycin, and L-15 medium (Grand Island Biological Co., Grand Island, N. Y.); fetal calf serum (Flow Laboratories, Inc., Rockville, Md.); 1-300 trypsin (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio); 16-mm well tissue culture trays and 96-well cloning trays (Linbro Scientific Co., New Haven, Conn.); C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine); rabbit anti-mouse kappa IgG, goat anti-rabbit IgG, and goat anti-mouse IgG (Gateway Immunosera Co., Cahokia, Ill.); Gelvatol 20/30 (Monsanto Co., St. Louis, Mo.); FITC, crystalline DCC-treated trypsin, twice crystallized papain, and twice crystallized pepsin (Sigma Chemical Co., St. Louis, Mo.); KLH (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), and [^{125}I]sodium iodide, carrier free, and [5-^{125}I]iododeoxyuridine, >2,000 Ci/nmol (New England Nuclear, Boston, Mass.). Rabbit C′ was from freshly prepared rabbit serum and was stored frozen at −70°C.

Results

Selection of Variants. Variants with altered binding properties for IgG were selected by treating cells with rabbit C′ after incubation with soluble complexes of an IgG2a myeloma protein, LPC-1, and rabbit anti-κ. IgG2a binds tightly with a K_m of 7 μg/ml at 37°C to mouse macrophages (8), and the concentration of IgG2a used in the selection protocol was 15 μg/ml. At this concentration of IgG2a there was maximum cytotoxicity when 10 μg/ml of rabbit anti-κ was added. When 20% rabbit C′ was used, 98–99% of the initial cells were killed. The specificity of the selection (Table I) shows cytotoxicity obtained with various myeloma proteins and rabbit anti-κ in the presence of 5% rabbit C′. Only the IgG2a myeloma protein LPC-1 and, to a lesser extent, the IgG2b myeloma protein MPC-11, which is also cytophilic (7, 9), kill cells in the presence of rabbit anti-κ and C′.

Variant lines were isolated from 5 to 10 × 10^6 initial cells using four cycles of selection as described in the Materials and Methods. The frequency of resistant clones varied from 10^{-4} to 10^{-7} for different subclones of $P388D_1$. When retested, the isolated clones exhibited varying degrees of resistance to the selection used. One clone, 3.42A, which grew particularly well in culture was selected for further study. This clone differs from the parent line, 3.4, in that the cells contain distinctive large phase-lucent granules in the perinuclear region, and the cells do not adhere as tightly to tissue culture dishes. Fig. 1 shows the sensitivity of 3.42A and of the parent, 3.4, to preformed LPC-1 rabbit anti-κ complexes and to a rabbit anti-$P388D_1$ macrophage plasma membrane antiserum. The variant and parent are equally sensitive to anti-$P388D_1$ plasma membrane antiserum and C′, so the resistance of the variant to rabbit C′ and IgG2a-rabbit anti-κ complexes is not due to resistance to the C′-mediated lysis per se.

Immunofluorescence. C′ fixation leading to cell lysis in the selection procedure is presumably mediated by the rabbit antibody. Because two IgG molecules have to be adjacent to fix C′ (31), there can be a disproportionate change in cytotoxicity with small differences in the amount of rabbit IgG bound (32). The immunofluorescence experiment was designed to examine the amount of rabbit

TABLE I
Cytotoxicity to P388D$_1$ Cells of Myeloma Protein Rabbit Anti-κ Complexes and Rabbit C'

Proteins	Specific release
	%
Rabbit C'	0
Rabbit anti-κ, 10 μg/ml	2
LPC-1 (IgG2a), 15 μg/ml	−2
LPC-1 Fab, 15 μg/ml, + rabbit anti-κ, 10 μg/ml	0
LPC-1, 15 μg/ml, + rabbit anti-κ, 10 μg/ml	76
MPC-11 (IgG2b), 15 μg/ml, + rabbit anti-κ, 10 μg/ml	34
MOPC-21a (IgG1), 15 μg/ml, + rabbit anti-κ, 10 μg/ml	3

P388D$_1$ cells (3×10^5) in 16-mm wells were labeled with ^{125}IUDR, washed with HBSS, and incubated with proteins in 1:1 PBS-BSA:L-15 containing 5% rabbit C' at 37°C for 45 min. Cytotoxicity was calculated as described in the Materials and Methods. Spontaneous release was 20%.

IgG bound to the parent and variant clones under the conditions of the selection procedure. Incubated with complexes and stained with FITC-goat anti-rabbit IgG, the 3.42A cells showed weak fluorescence compared to the parent line (Figs. 2 A and B). When the two lines were stained with FITC-goat anti-mouse IgG after incubation with monomeric IgG2a, there were bright fluorescent patches on both cells (Figs. 2 C and D). This suggested that the resistance of the variant to rabbit anti-κ-LPC-1 complexes was due to reduced binding of rabbit IgG and not to absence of the receptor for IgG2a.

Binding of Mouse IgG2a and Rabbit Anti-DNP IgG. The binding of monomeric IgG2a and rabbit IgG to the parent and variant lines was then studied in more detail. Monomeric rabbit IgG binds to mouse macrophages very weakly, exceeding the background binding to 3T3 cells by two fold at best. In order to do experiments on the binding of antigen-antibody complexes, rabbit anti-DNP antibody was isolated by affinity chromatography. The binding of labeled anti-DNP IgG was studied with varying ratios of DNP-BSA to anti-DNP IgG (Fig. 3). The optimum molar ratio of DNP-BSA:IgG for binding was 1.3 for $DNP_{2.4}BSA$ and 0.38 for $DNP_{10.9}BSA$. There was less binding of the anti-DNP IgG in both antibody and antigen excess. The amount of rabbit IgG bound to the 3.42A variant was less in all cases than that bound to the parent line 3.4 (Fig. 3).

Complexes of rabbit anti-DNP with $DNP_{2,4}BSA$ and $DNP_{10.9}BSA$ were soluble over a wide range of antigen concentrations, even when IgG was present at a concentration of 1 mg/ml. To confirm that the complexes were binding via the Fc domain the $F(ab')_2$ fragment of the rabbit anti-DNP IgG was prepared and iodinated. The $F(ab')_2$ had the same hemagglutination titer as the native IgG, but complexes of $DNP_{10.9}BSA$ with the $F(ab')_2$ fragment did not bind to either cell (Fig. 3 B).

The binding of monomeric IgG2a and of complexes of rabbit anti-DNP IgG DNP-BSA (formed with $DNP_{2.4}BSA$ or $DNP_{10.9}BSA$ at the optimum ratio of antigen to antibody for binding) was studied with the parent line 3.4 and the variant 3.42A. The binding data were corrected for a nonspecific component by subtraction of values obtained for NIH-3T3 cells (usually 0.1–0.2% of input radioactivity), and the data were plotted according to $r/c = K_a n - K_a r$ (Scat-

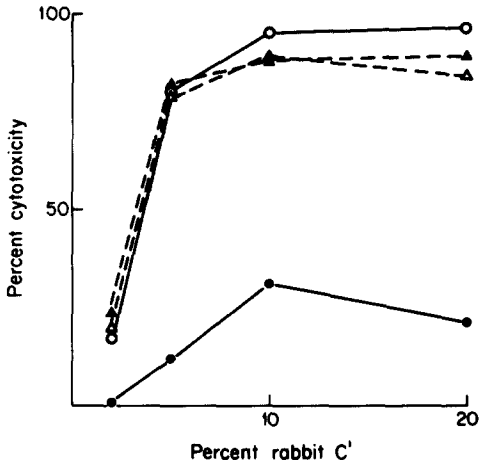

FIG. 1. Cytotoxicity of IgG2a rabbit anti-κ complexes or rabbit anti-C57BL/6 thioglycollate-induced macrophage plasma membrane antiserum and rabbit C'. 3.4 or 3.42A cells were labeled with ^{125}IUDR as described in the Materials and Methods. The cells were incubated at 37°C for 45 min with rabbit C' as indicated and either LPC-1 rabbit anti-κ complexes (15 μg/ml LPC-1, 10 μg/ml rabbit anti-κ) preformed by incubation at 4°C for 20 min, or at 1:800 dilution of the rabbit anti-macrophage plasma membrane antiserum in 1:1 PBS-BSA:L-15. Spontaneous release for both 3.4 and 3.42A was 20%. (●—●), 3.42A, LPC-1 rabbit anti-κ complexes; (○—○), 3.42A, rabbit anti-macrophage plasma membrane; (△---△), 3.4, LPC-1 rabbit anti-κ complexes; and (▲---▲), 3.4, rabbit anti-macrophage plasma membrane.

chard equation), where K_a is the equilibrium constant (in M^{-1}), r is micrograms of IgG bound per well, c is unbound Ig in M, and n is the limiting value for r as c becomes very large. Fig. 4 shows the Scatchard plot for the binding of IgG2a to the parent and variant lines; the Ka for both was the same (Ka = 1.1×10^8 M^{-1}) and the number of binding sites per cell was comparable (Table II). A large number of independently derived variant clones of P388D$_1$ were tested for binding of IgG2a and all of them bound IgG2a with the same affinity.

The binding at 4°C and 37°C of DNP$_{2.4}$BSA anti-DNP IgG complexes formed at a molar ratio of DNP$_{2.4}$BSA:IgG of 1.3 (Fig. 3 A) is shown in Fig. 5; Ka values and numbers of sites per cell are tabulated for DNP$_{2.4}$BSA and DNP$_{10.9}$BSA complexes in Table II. Comparison of the Scatchard plots for binding of the rabbit antigen-antibody complexes to 3.4 and 3.42A lines showed striking differences. The number of high affinity sites in the variant line was 10% that of the parent. Not only the number of sites per cell, but also the affinity of those binding sites was altered in the variant. Examination of data from comparable experiments shows that the Ka of the rabbit IgG complexes for the variant was always threefold higher than the parent. At both 4°C and 37°C there was a discontinuity in slope of the data plotted for the 3.42A line (Fig. 5), suggesting that there were two binding sites, high and low affinity, which are tabulated as Ka$_1$ and Ka$_2$ in Table II. The binding to the low affinity site might have been masked in the data for the parent line, 3.4, because of the large number of high affinity binding sites. Though both the number of sites per cell and the Ka of the high affinity site for rabbit IgG in antigen-antibody complexes were altered in

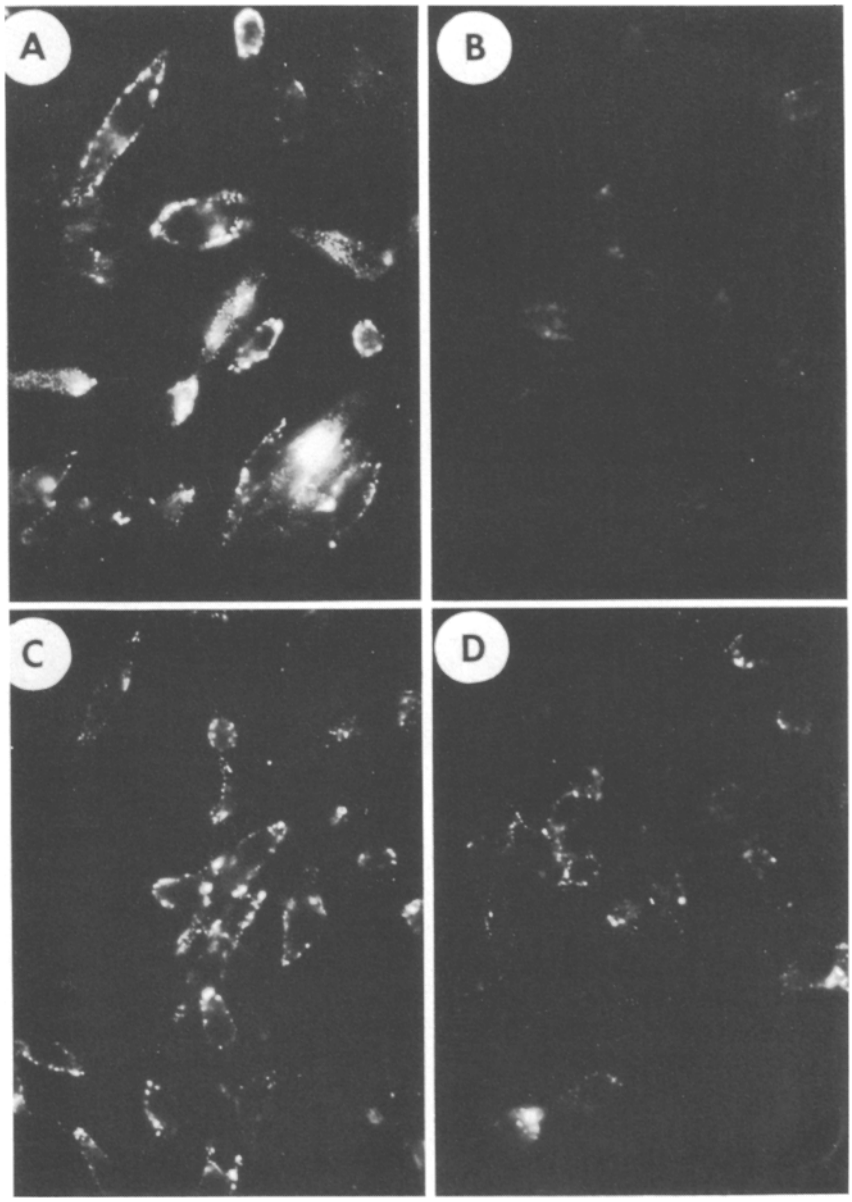

FIG. 2. Immunofluorescence to demonstrate binding of rabbit antigen-antibody complexes and monomeric mouse IgG2a to 3.4 and 3.42A cells. (A) 3.4 cells and (B) 3.42A cells incubated for 45 min at 37°C with IgG2a rabbit anti-κ complexes, stained with FITC-goat anti-rabbit IgG: (C) 3.4 cells, and (D) 3.42A cells incubated with LPC-1 stained with FITC-goat anti-mouse IgG.

the variant, the binding of monomeric IgG2a was unchanged in both respects (Fig. 3 and Table II).

The size of the complexes formed with $DNP_{10.9}BSA$ and $DNP_{2.4}BSA$ as antigens was probably different since the optimum ratios of antigen to antibody for

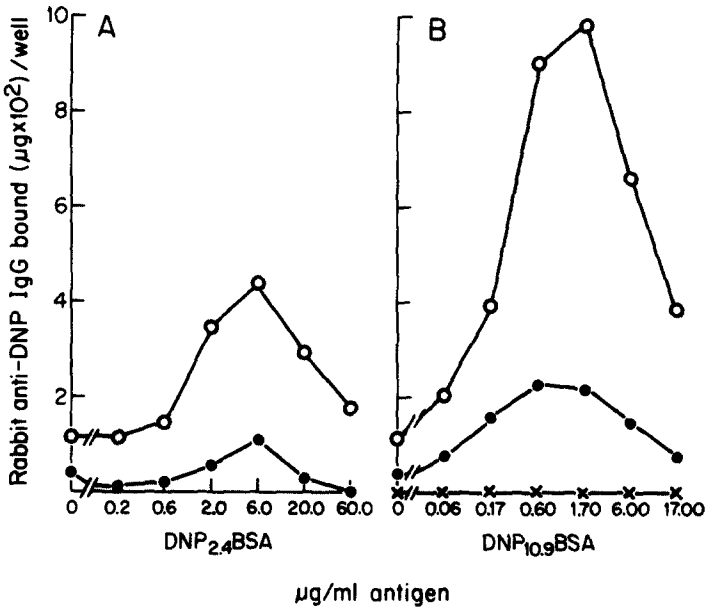

FIG. 3. Binding to 3.4 and 3.42A cells of rabbbit anti-DNP IgG in relation to DNP-BSA. Cells (3.5×10^5) were plated in 16-mm wells. Complexes were preincubated for 2 h at 4°C and contained $DNP_{2.4}BSA$ (A) or $DNP_{10.9}BSA$ (B) as noted, 10 μg/ml of rabbit anti-DNP IgG, and ^{125}I-rabbit anti-DNP (40 ng/ml, 5×10^3 cpm/ng) in 1:1 PBS-BSA:L-15. (○—○), 3.4 cells; (●—●), 3.42A cells; (×—×), 3.4 cells, rabbit anti-DNP $F(ab')_2$ fragment.

binding were different, 0.38 and 1.3, respectively (Fig. 3). Reflecting these differences, the Ka values of the $DNP_{10.9}BSA$ complexes were higher than the corresponding values of the $DNP_{2.4}BSA$ complexes. However, since the number of sites per cell binding the labeled rabbit IgG determined with complexes of either size agreed quite well, these values may be valid. The binding of the complexes appears to be an exothermic reaction, the Ka values being higher at 4°C than at 37°C. However, these differences may reflect changes in aggregate size with temperature and should be interpreted cautiously.

Trypsin Sensitivity of Fc Receptors. The receptor for mouse IgG2a was previously shown to be sensitive to treatment of the macrophages with trypsin (8, 18). The binding of rabbit anti-DNP $DNP_{10.9}BSA$ complexes to both the 3.4 line and thioglycollate-stimulated C57BL/6 macrophages was examined with respect to its sensitivity to the action of trypsin. The results showed almost total inhibition of binding of IgG2a in both cell types (Fig. 6 A) but no inhibition of binding of the antigen-antibody complexes to either cell (Fig. 6 B). The thioglycollate-stimulated macrophages have about threefold the number of Fc receptors per cell for monomeric IgG2a as the $P388D_1$ line, and an equal number of receptors for rabbit IgG in antigen-antibody complexes.

Rosette Formation with SRBC. The difference in trypsin sensitivity of the Fc receptors for IgG2a and rabbit antigen-antibody complexes were further investigated by studying rosette formation with either SRBC to which IgG was fixed by glutaraldehyde, or SRBC which were derivatized lightly with TNP and then incubated with a nonhemagglutinating concentration of rabbit anti-DNP IgG.

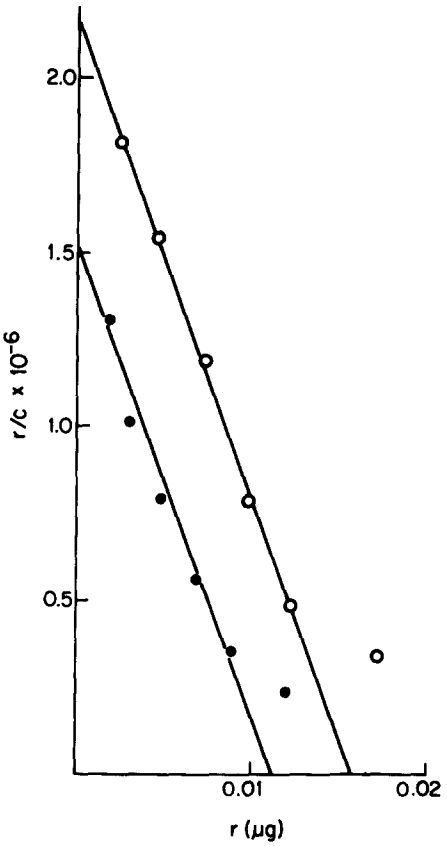

FIG. 4. The binding of LPC-1 to 3.4 and 3.42A cells. 3.4 cells (3.5×10^5) or 3.42A cells (3.1×10^5) in 16-mm wells were incubated at 4°C for 60 min with a constant amount of ^{125}I-LPC-1 (40 ng/ml, 5×10^3 cpm/ng) and varying amounts of unlabeled LPC-1. r, micrograms of LPC-1 bound per well; c, unbound concentration of LPC-1 in moles per liter. (○—○), 3.4 cells; and (●—●), 3.42A cells.

Treatment of the 3.4 line with trypsin (Fig. 7 B) abolished rosette formation with IgG2a-coated SRBC as seen in Fig. 7 A. The variant line, 3.42A (Fig. 7 C) formed rosettes with IgG2a-coated SRBC as well as the parent line. Binding was specific since SRBC coated with a noncytophilic IgG1 myeloma protein, MOPC-21a (Fig. 7 D), or the F(ab) fragment of LPC-1, an IgG2a myeloma protein (not shown), did not form rosettes.

TNP-coated SRBC incubated with rabbit anti-DNP IgG and then washed formed dense rosettes with the 3.4 line (Fig. 7 E), and, unlike the observation with IgG2a-coated SRBC, rosette formation was unaffected by trypsinization (Fig. 7 F). Although there were some rosettes on the variant line 3.42A (Fig. 7 G), the number of SRBC per cell was much less than the number on the parent line 3.4—the outlines of the cell bodies and pseudopodia were always visible and many cells did not form rosettes at all. Trypsinization did not affect formation of rosettes with IgG-coated TNP-SRBC on the 3.42A line (not shown). Controls of TNP-SRBC and TNP-SRBC coated with the $F(ab')_2$ fragment of the rabbit anti-DNP IgG did not form rosettes.

TABLE II

Association Constants and Number of Sites Per Cell for Binding of LPC-1 and Rabbit Anti-DNP DNP-BSA Complexes to 3.4 and 3.42A Cells

Protein	Temperature (°C)	Cell type: 3.4 Ka ($M^{-1} \times 10^{-7}$)	3.4 Sites per cell ($\times 10^{-5}$)	3.42A Ka ($M^{-1} \times 10^{-7}$)	3.42A Sites per cell ($\times 10^{-5}$)
LPC-1	4	11 ± 1.0	2.1 ± 0.4	11 ± 1	1.7 ± 0.4
Rabbit anti-DNP $DNP_{2.4}$ BSA	4	1.7 ± 0.2	8.7 ± 2	Ka_1 = 6.0 ± 0.8 Ka_2 = 0.14 ± 0.02	1.1 ± 0.2 7.6 ± 1
Rabbit anti-DNP $DNP_{10.9}$ BSA	4	3.3 ± 0.2	8.6 ± 2	9.5 ± 0.9	1.0 ± 0.2
Rabbit anti-DNP $DNP_{2.4}$ BSA	37	0.72 ± 0.06	4.6 ± 1	Ka_1 = 2.1 ± 0.8 Ka_2 = 0.12 ± 0.04	0.38 ± 0.07 2.8 ± 0.6
Rabbit anti-DNP $DNP_{10.9}$ BSA	37	2.5 ± 0.2	4.9 ± 1	6.0 ± 1	0.68 ± 0.2

Assays were performed as detailed in Figs. 4 and 5. $DNP_{10.9}$ BSA rabbit anti-DNP complexes were formed at an antigen to antibody ratio of 0.38.

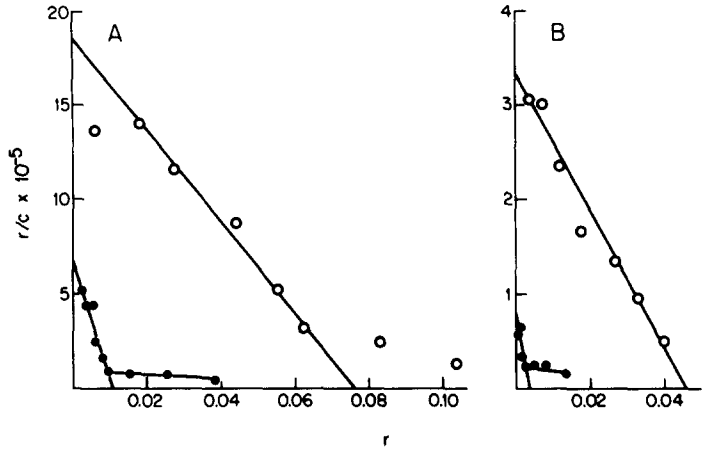

FIG. 5. The binding of $DNP_{2.4}$BSA rabbit anti-DNP complexes to 3.4 and 3.42A cells. 3.4 and 3.42A cells (4×10^5) and NIH-3T3 cells (4×10^4) in 16-mm wells were incubated at 4°C (A) or 37°C (B) with increasing amounts of $DNP_{2.4}$BSA rabbit anti-DNP complexes formed at an antigen-antibody ratio of 1.3. The specific activity of the ^{125}I-rabbit anti-DNP IgG was 4×10^5 cpm/μg. Data were corrected for background binding to NIH-3T3 cells. For r and c see Fig. 4. (○—○), 3.4 cells; and (●—●), 3.42A cells.

The TNP-SRBC complexed with rabbit anti-DNP IgG bound to a trypsin-resistant Fc receptor, which I assumed would also be the case for rabbit IgG in the absence of antigen. However, SRBC coupled with rabbit IgG using glutaraldehyde did not form rosettes with trypsinized 3.4 cells.

Rosette Formation with Sephadex G-25 Beads. To test further the hypothesis that formation of immune complexes by rabbit IgG determines the receptor to which that IgG can bind, another method of coupling IgG to particles besides glutaraldehyde fixation was sought. Superfine Sephadex G-25 beads (10–40 μm)

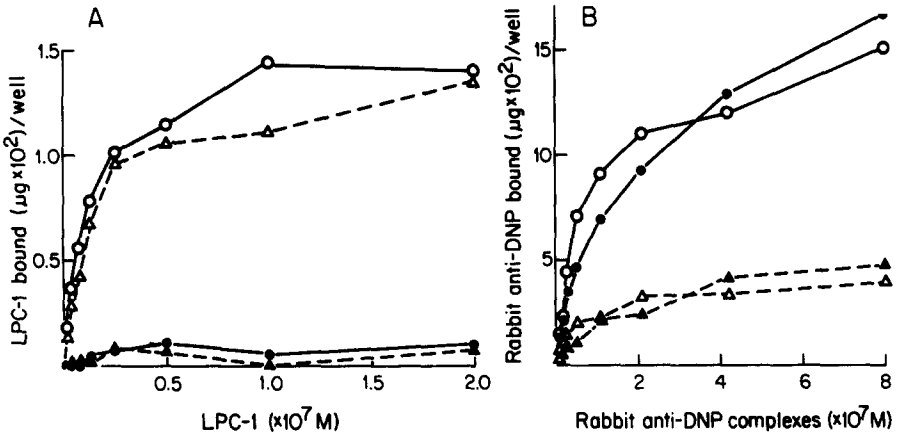

FIG. 6. Sensitivity to trypsinization of binding of IgG2a and rabbit antigen-antibody complexes to P388D$_1$ cells and thioglycollate-induced macrophages. 3.4 cells (5×10^5), thioglycollate-induced C57BL/6 macrophages (2.1×10^5) and NIH-3T3 cells (3×10^4) in 16-mm wells were incubated either before or after trypsin treatment with ^{125}I-LPC-1 (A) as described in Fig. 4, or with DNP$_{10.9}$BSA ^{125}I-rabbit anti-DNP complexes (B) formed at an antigen to antibody ratio of 0.38, as described in Fig. 5. (○ – ○), 3.4 cells; (● – ●), trypsin-treated 3.4 cells; (△---△), thioglycollate-induced macrophages; and (▲---▲), trypsin-treated thiglycollate-induced macrophages.

were not bound rapidly by macrophages in serum-free medium, and could be readily activated using cyanogen bromide. The coupling was performed in PBS at pH 7.4 which does not diminish the antigen-binding capacity of the bound IgG (25).

Rosettes formed with Sephadex G-25 beads are shown in Fig. 8. The DNP$_{10.9}$BSA-Sephadex to which rabbit anti-DNP IgG was bound formed rosettes with both normal 3.4 (Fig. 8 A), and trypsinized cells (Fig. 8 B). Sephadex beads to which the same anti-DNP IgG was coupled with cyanogen bromide (no antigen present) bound only to normal 3.4 cells (Fig. 8 D) and not at all to trypsinized cells (Fig. 8 E). The same binding pattern was seen using Sephadex to which IgG2a was coupled with cyanogen bromide (Figs. 8 G and H). Monomeric rabbit IgG bound weakly to mouse macrophages but when presented in multimeric form the IgG coupled to the Sephadex beads promoted avid binding. It is thus particularly striking that such Sephadex beads failed to bind to trypsinized P388D$_1$ cells.

Discussion

The presence of Fc receptors on macrophages, and the role of the Fc receptor in the recognition and ingestion of antibody-coated particles (33–35) have been well established. The results described in this paper demonstrate that the P388D$_1$ macrophage cell line and normal mouse peritoneal macrophages have two Fc receptors which differ in the Ig's they bind and in their sensitivity to trypsinization.

Genetic evidence for two Fc receptors comes from the isolation of a stable variant of a clone of the P388D$_1$ line. The binding of monomeric IgG2a and rabbit IgG in antigen-antibody complexes was examined by immunofluorescent tech-

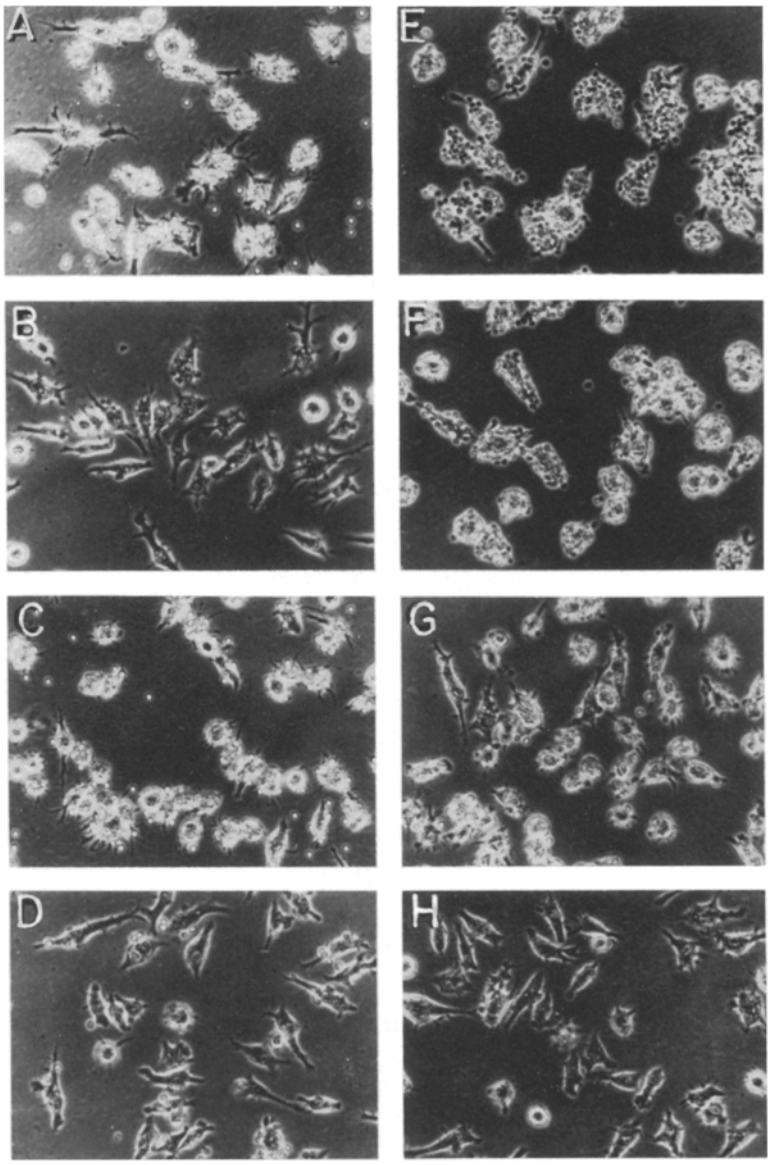

FIG. 7. SRBC rosettes on 3.4, trypsin-treated 3.4, and 3.42A cells. Cells (2×10^5) plated on 60-mm dishes were trypsinized, SRBC suspended in 1:1 PBS-BSA:L-15 (2 ml, 5×10^7 SRBC/ml) were added, and the dishes were incubated at 37°C for 15 min with intermittent rocking. The nonadherent SRBC were removed by repeated dipping of the dishes in PBS and the cells were fixed with 1% glutaraldehyde in PBS. (A) 3.4 cells, UPC-10-coated SRBC; (B) trypsin-treated 3.4 cells, UPC-10-coated SRBC; (C) 3.42A cells, UPC-10-coated SRBC; (D) 3.4 cells, MOPC-21a-coated SRBC; (E) 3.4 cells, anti-DNP IgG-coated TNP-SRBC; (F) trypsin-treated 3.4 cells, anti-DNP IgG-coated TNP-SRBC: (G) 3.42A cells, anti-DNP-coated TNP-SRBC; and (H) 3.4 cells, TNP-SRBC.

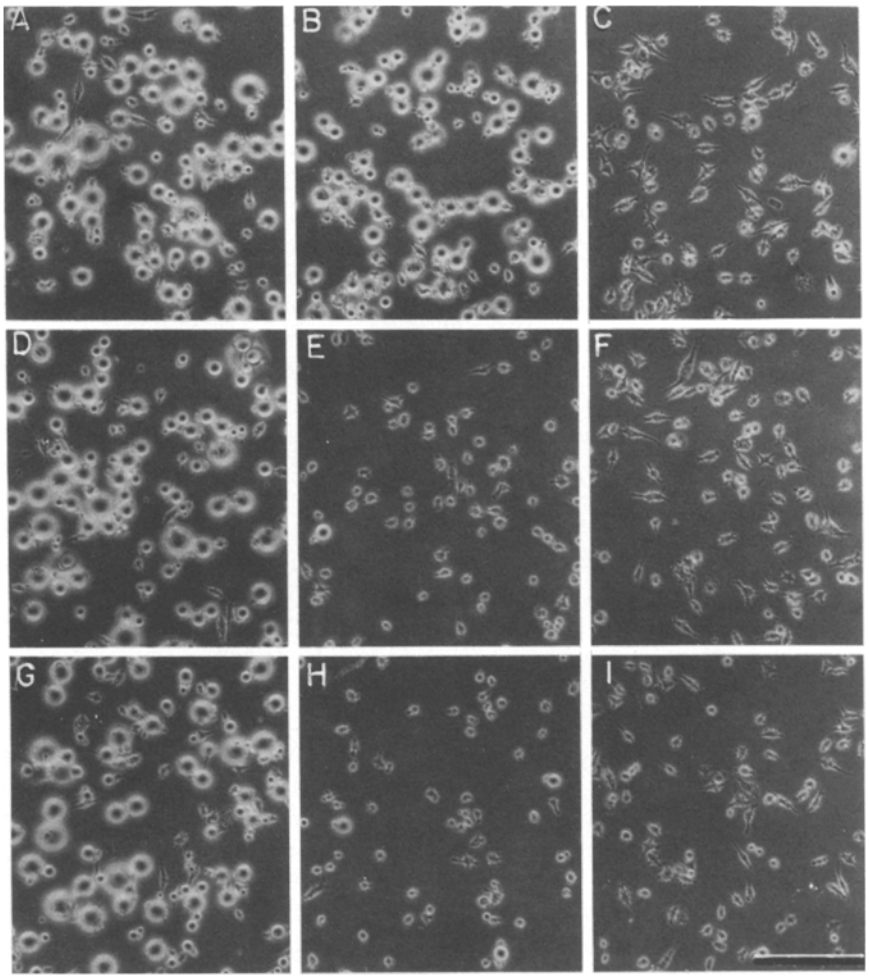

FIG. 8. Binding of protein-coated Sephadex G-25 beads to 3.4 and trypsinized 3.4 cells. Sephadex G-25 beads coated with proteins were resuspended at a concentration of 0.03 g wet weight of Sephadex/ml in 1:1 PBS-BSA:L-15 and 2 ml added to cells (5×10^5) in 60-mm dishes. After incubation at 37°C for 15 min with intermittent rocking the nonadherent beads were washed in PBS, and the cells were fixed as before. (A) 3.4 cells, $DNP_{10.9}BSA$-Sephadex incubated with rabbit anti-DNP IgG; (B) trypsin-treated 3.4 cells, $DNP_{10.9}BSA$ Sephadex incubated with rabbit anti-DNP IgG; (C) 3.4 cells, $DNP_{10.9}BSA$ Sephadex; (D) 3.4 cells, rabbit anti-DNP IgG Sephadex; (E) trypsin-treated 3.4 cells, rabbit anti-DNP IgG Sephadex: (F) 3.4 cells, BSA Sephadex incubated with rabbit anti-DNP IgG; (G) 3.4 cells, LPC-1 Sephadex; (H) trypsin-treated 3.4 cells, LPC-1 Sephadex; and (I) 3.4 cells, BSA Sephadex. The bar is 200 μm.

niques and by direct binding studies with iodinated IgG, and in both cases there were striking differences between the parent and variant clones. However, the binding of IgG2a to the two cell lines was comparable. In these experiments only surface IgG released by treatment with trypsin was measured. There may have been some phagocytosis of antigen-antibody complexes at 37°C, since the number of sites at saturation was somewhat less than the value determined at 4°C,

but the number of sites determined using complexes formed with $DNP_{2.4}BSA$ and $DNP_{10.9}BSA$ agree.

Receptors binding IgG2a and rabbit IgG in complexes could also be differentiated on the basis of trypsin sensitivity. The receptor that bound mouse IgG2a, whether studied with monomeric IgG2a, SRBC coupled with IgG2a using glutaraldehyde, or Sephadex G25 beads coupled with IgG2a via cyanogen bromide activation, was sensitive to trypsinization. In agreement with other studies (5, 36), the receptor that bound rabbit antibody-antigen complexes was trypsin resistant. Walker (9) reported that binding of both IgG2a and IgG2b was trypsin-resistant in the SV-40-derived macrophage line IC-21. Since similar results, with respect to trypsin sensitivity of the IgG2a Fc receptor were obtained with $P388D_1$ and thioglycollate-stimulated macrophages, the IC-21 line may be less "normal" in this respect.

The presence of multiple Fc receptors on mouse macrophages has been reported previously (16–18). Cytophilic antibody in "early" mouse antisera binds to a trypsin-sensitive Fc receptor, whereas hyperimmune antibody binds to a trypsin-resistant receptor. Walker (9) suggested there were separate receptors for mouse IgG2a and IgG2b because aggregated IgG2b binding could not be inhibited by monomeric IgG2a and vice versa, and IgG2b could not be shown to bind as a monomer. We found that at least one IgG2b myeloma protein did bind as a monomer, albeit with a Ka at 4°C of $7 \times 10^6\ M^{-1}$, 1/20 the Ka of IgG2a at 4°C (8).

Of particular interest is the observation that, unlike rabbit IgG in antigen-antibody complexes, uncomplexed rabbit IgG binds to a trypsin-sensitive Fc receptor. Sephadex beads coated with rabbit anti-DNP IgG using cyanogen bromide activation did not bind to trypsinized cells but DNP-BSA Sephadex beads to which the same anti-DNP IgG was bound formed rosettes regardless of trypsin treatment (Fig. 8). Similar results were found with SRBC coated with rabbit IgG using glutaraldehyde and TNP-SRBC coated with anti-DNP IgG (Fig. 7). These results could be due to alteration in conformation of the Fc domain upon binding of antigen (37, 38), or to fundamental differences in the "multimeric" presentation of the Fc domains in antigen-antibody complexes compared to Sephadex beads with antibody coupled to them using cyanogen bromide.

The demonstration of two classes of receptors on mouse cells raises the issue of whether there might also be multiple receptors on macrophages of other species, which have been overlooked because of lack of differential sensitivity to trypsin. If different classes of macrophages expressed different densities of one or another Fc receptor on their surfaces, this might lead to differences in function. Indeed, there have been reports of heterogeneity in rosette formation by guinea pig (39) and rabbit macrophages (40). The physiological function of the multiple subclasses of mouse IgG is obscure. Mouse IgG1 antibodies suppress an immune response to SRBC, while IgG2 has the opposite feedback effect (41), but the locus of action of these different antibodies is unknown. The subclass of mouse IgG which is specific for each receptor and the effect of formation of antigen-antibody complexes in each case remain to be investigated. The selection of suitable variant macrophage lines should prove useful in this respect, and might also help in studies of macrophage function.

Summary

A stable variant of a clone of the $P388D_1$ macrophage line was isolated using four cycles of treatment with mouse IgG2a-rabbit anti-κ complexes and rabbit complement. The variant had the same Ka and about the same number of sites per cell for IgG2a as the parent line. However, the variant had 10% as many binding sites for rabbit IgG in soluble antigen-antibody complexes, and the affinity of binding was threefold higher. This change in binding of complexes to cells of a cloned line without alteration of IgG2a binding provides evidence for the presence of two distinct Fc receptors.

The two receptors could also be distinguished on the $P388D_1$ line and on thioglycollate-induced mouse peritoneal macrophages by differential sensitivity to trypsinization. The receptor that binds monomeric IgG2a, sheep erythrocytes (SRBC) covalently bound with IgG2a or rabbit IgG using glutaraldehyde, and Sephadex beads coupled with IgG2a or rabbit IgG using cyanogen bromide activation, is sensitive to trypsinization. The receptor that binds soluble rabbit antibody-antigen complexes, trinitrophenyl-SRBC and dinitrophenyl(DNP)-bovine serum albumin Sephadex beads coated with rabbit anti-DNP IgG is trypsin resistant. The observation that uncomplexed rabbit IgG does not bind to the trypsin-resistant receptor, whereas the same IgG bound to its antigen does, suggests that conformational changes induced by the binding of ligand may be of consequence in macrophage function.

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References

1. Howard, J. G., and B. Benacerraf. 1966. Properties of macrophage receptors for cytophilic antibodies. *Br. J. Exp. Pathol.* 47:193.
2. Lesile, R. G. Q., and S. Cohen. 1974. Cytophilic activity of IgG2 from sera of unimmunized guinea pigs. *Immunology.* 27:577.
3. Arend, W. P., and M. Mannik. 1972. In vitro adherence of soluble immune complexes to macrophages. *J. Exp. Med.* 136:514.
4. LoBuglio, A. F., R. S. Cotran, and J. H. Jandl. 1967. Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. *Science (Wash. D. C.).* 158:1582.
5. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of particulate horseradish peroxidase (HRP)-anti HRP immune complexes with mouse peritoneal macrophages. *J. Cell Biol.* 55:616.
6. Koren, H. S., B. S. Handwerger, and J. R. Wunderlich. 1975. Identification of macrophage-like characteristics in a cultured murine tumor line. *J. Immunol.* 114:894.
7. Ralph, P., J. Prichard, and M. Cohn. Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. *J. Immunol.* 114:898.
8. Unkeless, J. C., and H. N. Eisen. 1975. Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. *J. Exp. Med.* 142:1520.
9. Walker, W. S. 1976. Separate Fc-receptors for immunoglobulins IgG2a and IgG2b on an established cell line of mouse macrophages. *J. Immunol.* 116:911.

10. Henson, P. M. 1969. The adherence of leukocytes and platelets induced by fixed IgG antibody or complement. *Immunology*. **16**:107.
11. Basten, A., J. F. A. P. Miller, J. Sprent, and J. Pye. 1971. A receptor for antibody on B lymphocytes. I. Method of detection and functional significance. *J. Exp. Med.* **135**:610.
12. Dickler, H. B., and H. G. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. *J. Exp. Med.* **136**:191.
13. Anderson, C. L., and H. M. Grey. Receptors for aggregated IgG on mouse thymocytes. *J. Exp. Med.* **139**:1175.
14. Tigelaar, R. E., N. M. Vaz, and Z. Ovary. 1971. Immunoglobulin receptors on mouse mast cells. *J. Immunol.* **106**:661.
15. Watkins, J. F. 1964. Adsorption of sensitized sheep erythrocytes to HeLa cells infected with Herpes Simplex Virus. *Nature (Lond.)*. **202**:1364.
16. Nelson, D. S., S. Kossard, and P. E. Cox. 1967. Heterogeneity of macrophage cytophilic antibodies in immunized mice. *Experientia (Basel)*. **23**:490.
17. Kossard, S., and D. S. Nelson. 1968. Studies on cytophilic antibodies. IV. The effects of proteolytic enzymes (trypsin and papain) on the attachment to macrophages of cytophilic antibodies. *Aust. J. Exp. Biol. Med. Sci.* **46**:63.
18. Askenase, P. W., and B. J. Hayden. 1974. Cytophilic antibodies in mice contact-sensitized with oxazolone. *Immunology*. **27**:563.
19. Dawe, C. J., and M. Potter. 1957. Morphologic and biologic progression of a lymphoid neoplasm of the mouse in vivo and in vitro. *Am. J. Pathol.* **33**:603.
20. Rabinovitch, M., and M. J. DeStephano. 1976. Cell shape changes induced by cationic anesthetics. *J. Exp. Med.* **143**:290.
21. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139**:834.
22. Sonoda, S., and M. Schlamowitz. 1970. Studies of ^{125}I trace labeling of immunoglobulin G by chloramine T. *Immunochemistry*. **7**:885.
23. Little, J. R., and H. N. Eisen. 1967. Preparation of immunogenic 2,4-dinitrophenyl and 2,4,6-trinitrophenyl proteins. *Methods Immunol. Immunochem.* **1**:128.
24. Eisen, H. N., W. Gray, J. R. Little, and E. S. Simms. 1967. Purification of antibodies to the 2,4-dinitrophenyl (DNP) group and the 2,4,6-trinitrophenyl (TNP) group. *Methods Immunol. Immunochem.* **1**:351.
25. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* **245**:3059.
26. Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of the digestion products. *Methods Med. Res.* **10**:134.
27. Klein, G., and P. Perlmann. 1963. *In vitro* cytotoxic effect of isoantibody measured as isotope release from labelled target cell DNA. *Nature (Lond.)*. **199**:451.
28. Cohen, A. M., J. R. Burdick, and A. S. Ketcham. 1971. Cell-mediated cytotoxicity: an assay using ^{125}I-iododeoxyuridine-labeled target cells. *J. Immunol.* **107**:895.
29. Avrameas, S., B. Taudou, and S. Chuilon. 1969. Glutaraldehyde, cyanuric chloride, and tetraazotized O-dianisidine as coupling reagents in the passive hemagglutination test. *Immunochemistry*. **6**:67.
30. Rittenberg, M. B., and K. L. Pratt. 1969. Antitrinitrophenyl plaque assay. Primary responses of BALB/C mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132**:575.
31. Anderson, B., H. Wigzell, and G. Klein. 1967. Some characteristics of 19S and 7S mouse isoantibodies in vivo and in vitro. *Transplantation (Baltimore)*. **5**:11.
32. Linscott, W. D. 1970. Effect of cell surface antigen density on immunological enhancement. *Nature (Lond.)*. **288**:824.

33. Rabinovitch, M. 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. *Exp. Cell Res.* **46**:19.
34. Griffin, F. M., Jr., C. Bianco, and S. C. Silverstein, 1975. Characterization of the macrophage receptor for the complement and demonstration of its functional independence from the receptor for the Fc portion of immunoglobulin G. *J. Exp. Med.* **141**:1269.
35. Griffin, F. M., Jr., J. A. Griffin, J. Leider, and S. C. Silverstein. 1975. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. *J. Exp. Med.* **142**:1263.
36. Walker, W. S., and A. Demus. 1975. Antibody-dependent cytolysis of chicken erythrocytes by an in vitro-established line of mouse peritoneal macrophages. *J. Immunol.* **114**:765.
37. Schlessinger, J., I. Z. Steinberg, D. Givol, J. Hochman, and I. Pecht. 1975. Antigen induced conformational changes in antibodies and their Fab fragments studied by circular polarization of fluorescence. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2775.
38. Huber, R., J. Deisenhofer, P. M. Colman, and M. Matsushima. 1976. Crystallographic structure studies of an IgG molecule and an Fc fragment. *Nature (Lond.).* **264**:415.
39. Rhodes, J. 1975. Macrophage heterogeneity in receptor activity: the activation of macrophage Fc receptor function in vivo and in vitro. *J. Immunol.* **114**:976.
40. Walker, W. S. 1974. Functional heterogeneity of macrophages: Subclasses of peritoneal macrophages with different antigen-binding activities and immune complex receptors. *Immunology.* **26**:1025.
41. Gordon, J., and R. A. Murgita. 1975. Suppression and augmentation of the primary in vitro immune response by different classes of antibodies. *Cell. Immunol.* **15**:392.