

# BINDING OF MONOMERIC IMMUNOGLOBULINS TO Fc RECEPTORS OF MOUSE MACROPHAGES\*

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The binding of certain immunoglobulins ("cytophilic" antibodies) to receptors on the surface of macrophages probably plays a central role in a number of macrophage functions, such as antibody-dependent cell-mediated cytotoxicity (1-3), macrophage "arming" (4,5), and possibly in the presentation of antigens to T and B cells in the induction of various immune responses. In this paper we studied the kinetics, equilibrium constants, and the specificity of the binding of various myeloma proteins by normal and thioglycollate-stimulated peritoneal macrophages and by a macrophage-like cell line, P388D<sub>1</sub> (1). The results demonstrate that proteins of the IgG2a class are bound with particularly high affinity to all of these cells. The rapid rates of association and dissociation of these proteins indicate that the macrophage surface is in rapid equilibrium with immunoglobulins (Igs) in the cell's immediate microenvironment. The macrophage receptors for Igs of this class are readily removed with trypsin and they can be regenerated by continued cultivation in the absence of serum protein.

## Materials and Methods

*Cell Culture.* P388D<sub>1</sub> cells, obtained from John Wunderlich (NIH, Bethesda, Md.) were grown in sealed spinners in alpha modified minimum Eagle's medium, (MEM) (6) with 10% fetal calf serum (FCS),<sup>1</sup> heat inactivated at 56°C for 30 min. Peritoneal macrophages were obtained from 20-30 g female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine); some of the mice were injected 4 days previously with 1 ml of thioglycollate medium, and peritoneal cells were collected as described (7). Cell yield from the thioglycollate-stimulated animals was  $4 \times 10^7$  cells/mouse, of which 70% were macrophages. Unstimulated mice yielded  $5 \times 10^6$  cells/mouse, of which 30% were macrophages; the remainder were mostly lymphocytes. Unstimulated and thioglycollate-stimulated cells were plated at densities of  $2 \times 10^6$  and  $7 \times 10^5$  cells, respectively, in 16-mm diameter tissue culture wells (Linbro Scientific Co., New Haven, Conn.), which were coated with purified bovine fibrinogen (8) at  $10 \mu\text{g}/\text{cm}^2$ ; fibrinogen (3 mg/ml) in phosphate-buffered saline (PBS) was diluted to 0.1 mg/ml in water, and 0.2-ml portions were used to coat the wells, which were then dried at 45°C. Normal macrophages, in particular, spread and adhered better on such dishes.

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<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; MEM, alpha modified minimal Eagle's medium; PBS, phosphate-buffered saline, 0.15 M NaCl-0.01 M K phosphate, pH 7.4; PBS-BSA, PBS containing 2 mg/ml of BSA.

P388D<sub>1</sub> macrophages were plated at densities from 2 to  $8 \times 10^5$  cells/well. Plates were maintained at 37°C in a 5% CO<sub>2</sub>-air mixture.

**Cell Counting.** Adherent macrophages cannot be dispersed with trypsin, and it was therefore difficult to enumerate the plated cells. Accordingly, we resorted to counting nuclei in cell lysates. After washing with PBS, cells were lysed with 0.5% Triton X-100 in PBS containing 5 mM EDTA. The lysate, containing dispersed nuclei, was diluted in 20 ml of PBS and counted in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) using a 140  $\mu$ m aperture. The results were in good agreement with the number of P388D<sub>1</sub> cells plated over a wide range of dilutions.

**Myeloma Proteins.** The following proteins of indicated subclasses were used: MOPC-104E, IgM; MOPC-21, IgG1; LPC-1 and 5563, IgG2a; MPC-11, IgG2b; and T-15, IgA. The corresponding tumors were obtained from Dr. Michael Potter, National Cancer Institute, Bethesda, Md., or from Littner Bionetics, Inc., Silver Springs, Md., and were maintained by serial passage as subcutaneous tumors in BALB/cJ and BALB/cAnN mice. Serum containing protein 5563 was a generous gift from Dr. George D. Sorenson, Dartmouth Medical School, Hanover, N. H. Ammonium sulfate was added to 45% of saturation to ascites fluid or serum from animals bearing the various tumors. Precipitates were dissolved in 150 mM NaCl, 10 mM Tris-HCl, pH 8, and dialyzed against the same buffer. After chromatography on Sephadex G-200, the 19S peak of the IgM protein (MOPC-104E) was purified by affinity chromatography on dextran (9). The other proteins were purified by ion-exchange chromatography on QAE-A50. MOPC-21 and MPC-11 (50 mg each) were dialyzed vs. 50 mM NaCl, 25 mM Tris-HCl, pH 7.5, loaded onto a QAE-A50 column (1.8  $\times$  29 cm), washed with two column volumes of the same buffer, and then eluted with 250 ml of a 0.05–0.7 M NaCl gradient in 25 mM Tris-HCl, pH 7.5. MOPC-21 eluted at 0.27 M NaCl, and MPC-11 at 0.43 M NaCl. LPC-1 and 5563 (50 mg each) were dialyzed vs. 50 mM NaCl, 25 mM Tris-HCl, pH 9.1, loaded onto a QAE-A50 column (1.8  $\times$  25 cm), and eluted with 250 ml of a 0.05–0.7 M NaCl gradient in 25 mM Tris-HCl, pH 9.1. Both proteins eluted at 0.34 M NaCl. Each of the proteins was dialyzed against PBS and stored at 4°C in the presence of 0.02% Na azide. To avoid aggregation the myeloma proteins were not frozen.

**Fc and Fab.** Purified LPC-1 (30 mg) in 10 ml of PBS containing 0.1 M 2-mercaptoethanol and 2 mM EDTA was incubated at 37°C with 0.34 mg papain (10). After 2 h no 7S Ig was present, as judged by chromatography on Sephadex G-200. After dialysis at 4°C against 50 mM NaCl, 50 mM Tris-HCl, pH 8.8, the reaction mixture was passed over a QAE-A50 column (0.8  $\times$  20 cm) in the same buffer: the Fab fraction was not retained, and the Fc fraction was eluted with 0.4 M NaCl, 50 mM Tris, pH 8.8. Both fractions were dialyzed against PBS and concentrated by vacuum dialysis. Of an initial 30 mg of LPC-1 the final yield was 8.5 mg Fc and 16.2 mg Fab, assuming the same extinction coefficient for the intact Ig and its fragments ( $E_{1\text{cm}}^{1\%} = 14.0$ ).

**Iodination.** Proteins were iodinated by a slight modification of the chloramine T method of Sonoda and Schlamowitz (11). Routinely, 50  $\mu$ g of protein were iodinated with 0.5 mCi of carrier-free [<sup>125</sup>I]Na (New England Nuclear, Boston, Mass.), and chloramine T at a 15-fold molar excess over the protein. The reaction was terminated after 30 min at 0°C by addition of 0.1 ml of a 0.04% tyrosine solution. After addition of 0.01 ml of 1 M KI, the reaction mixture was chromatographed on Sephadex G25 equilibrated with PBS containing 2 mg/ml of bovine serum albumin (PBS-BSA). This procedure usually resulted in 50% incorporation of the added <sup>125</sup>I, corresponding to 0.2 atoms <sup>125</sup>I per molecule of IgG. Increase in the amount of chloramine T to 50- or 100-fold molar excess improved the yield slightly (to ~80%), but resulted in denaturation, as evidenced by high backgrounds and nonlinearity in binding curves (see below). A trace amount of each iodinated protein (except 104E) in the presence of a large excess of the corresponding unlabeled myeloma protein was subjected to isoelectric focussing in 5% polyacrylamide slab gels containing 3 M urea and 2% ampholines, pH 3–10, according to Sakato et al.<sup>2</sup> Autoradiography revealed exact coincidence of the labeled and unlabeled protein, suggesting that there was little oxidation of cysteine to cysteic acid by the chloramine T. Iodinated proteins were stored at 4°C and used for no longer than 3 or 4 wk.

**Binding Assays.** Cultured cells in 16 mm tissue culture wells were washed twice with Hanks' balanced salt solution (HBSS) and incubated in serum-free MEM containing 0.05% lactalbumin hydrolysate for 2–12 h to allow dissociation or phagocytosis of membrane-bound Igs. The medium

<sup>2</sup> Sakato, N., S. Hall, and H. N. Eisen. Manuscript in preparation.

was then replaced with 0.19 ml of a 1:1 mixture of PBS-BSA and L-15 culture medium containing  $^{125}\text{I}$ -protein at the desired specific activity. A constant input of  $^{125}\text{I}$ -protein was used in all wells ( $\sim 8 \times 10^4$  cpm,  $0.02 \mu\text{g}$ ). After 30–60 min, the radioactive fluid was aspirated and the unbound Ig was rapidly removed by dipping the culture plate three times in 1.5 liters of ice-cold PBS. To count surface-bound  $^{125}\text{I}$ -protein, the cells were incubated with 0.4 ml of 0.25% "1-300" trypsin in PBS at  $37^\circ\text{C}$  for 20 min and the supernate was removed for assay. Neither the normal, the thioglycollate-stimulated, nor the P388D<sub>1</sub> macrophages were detached by the trypsin treatment. The background in empty wells or in wells containing mouse embryo fibroblasts as control cells was 0.2–0.5% of the input radioactivity; the same background was obtained when macrophages were incubated with the same amount of radiolabeled myeloma protein in the presence of a large excess of unlabeled myeloma protein (resulting in low specific activity,  $\leq 6 \times 10^5$  cpm/mg; see Fig. 3, below). All assays were in duplicate, and the variation from the mean usually did not exceed 10%.

Reagents were obtained and prepared as follows: FCS (Flow Laboratories, Inc., Rockville, Md.); L-15 culture medium (Grand Island Biological Co., Grand Island, N. Y.); MEM, alpha modified cell culture medium (K. C. Biological Inc., Lenexa, Kans.); fluid thioglycollate medium (Difco Laboratories, Detroit, Mich.); lactalbumin hydrolysate and trypsin 1-300 (Nutritional Biochemicals Corp., Cleveland, Ohio); crystallized trypsin, twice crystallized papain, and crystallized BSA (Sigma Chemical Co., St. Louis, Mo.); crystallized, TPCK-treated trypsin (Worthington Biochemical Corp., Freehold, N. J.); soybean trypsin inhibitor, component VI, and bovine fibrinogen, fraction I (Miles Laboratories Inc., Miles Research Div., Kankakee, Ill.); and ampholines, pH 3–10 (LKB, Stockholm, Sweden).

## Results

*Kinetics of Uptake and Dissociation of IgG2a.*  $^{125}\text{I}$ -LPC-1 ( $1.1 \mu\text{g/ml}$ ) was rapidly bound by P388D<sub>1</sub> macrophages (Fig. 1). The reaction followed pseudo-first order kinetics (Fig. 1 B), and at  $4^\circ\text{C}$  maximum binding by P388D<sub>1</sub> cells was achieved at 60 min (Fig. 1 A). Binding was more rapid at higher temperatures (Table I). In subsequent experiments, a 60 min incubation was used at  $4^\circ\text{C}$  and a

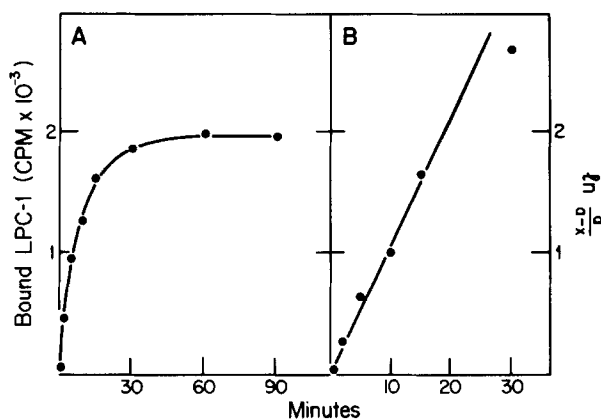


FIG. 1 Binding of an IgG2a myeloma protein (LPC-1) to a macrophage cell line (P388D<sub>1</sub>).  $5 \times 10^5$  macrophages were incubated at  $4^\circ\text{C}$  with LPC-1 ( $1.1 \mu\text{g/ml}$ ,  $5 \times 10^5$  cpm/ $\mu\text{g}$ ) for the indicated times, washed rapidly in cold ( $4^\circ\text{C}$ ) PBS, and treated with trypsin to release bound Ig. Data are corrected for nonspecific binding (i.e. counts per minute bound in empty wells or in wells with cultured fibroblasts, or to macrophage-containing wells treated with the standard amount of  $^{125}\text{I}$ -protein in the presence of 1 mg or more unlabeled myeloma protein). (A) Time-course of association. (B) Plot of data in A according to a first-order reaction (Ig in great excess). The value for  $a$  was the average counts per minute bound at 60- and 90-min time points in A;  $x$  was counts per minute bound at earlier time points.

TABLE I  
*Kinetic Constants for the Binding of an IgG2a Myeloma Protein (LPC-1) to a Macrophage Cell Line (P388D<sub>1</sub>)*

Temperature	Pseudo-first order association constant	First order dissociation constant	T <sub>1/2</sub> for dissociation
°C	(min <sup>-1</sup> )	(min <sup>-1</sup> )	(min)
4	0.11	0.083	8.4
24	0.22	0.21	3.3
37	0.44	0.26	2.6

Conditions were as described in Figs. 1 and 2. For measurements of dissociation rates the culture plates were washed in PBS at the same temperature at which the incubation was carried out (1st column at left), before addition of 0.5 ml of PBS-BSA.

30 min at other temperatures. Under the same conditions, the amount of LPC-1 bound by cultured mouse embryo fibroblasts and by empty wells was no more than about 5% of the maximal amount bound by the macrophages.

The dissociation of LPC-1 from macrophages was investigated by following release of the cell-bound Ig at various temperatures (Fig. 2 A). The dissociation obeyed first order kinetics (Fig. 2 B), and the rate increased with increasing temperature (Table I). The half times for dissociation (T<sub>1/2</sub>) obtained from the first order rate constants are summarized in Table I. The T<sub>1/2</sub> at 4°C, 8.4 min, is long compared to the time it takes to wash the plates free of nonbound Ig (15 sec); thus, it was not necessary to correct for dissociation of bound Ig when subsequent measurements were made of the rate or extent of binding. The linearity of the first order dissociation and pseudo-first order association kinetics suggest strongly that LPC-1 interacts with a single class of receptor molecules on the cell surface.

*Equilibrium Constant and Number of IgG2a Receptors Per Cell.* P388D<sub>1</sub> macrophages (8 × 10<sup>5</sup> cells per well) were incubated for 30–60 min with a constant amount of <sup>125</sup>I-LPC-1 and various amounts of unlabeled LPC-1. Fig. 3 shows a typical binding curve. As control, the binding to C57BL/6J embryo fibroblasts was also measured. To derive equilibrium (association) constants and number of binding sites per cell, the data were plotted according to  $r/c = K_a r - nr$  (Scatchard equation), where  $K_a$  is the equilibrium constant (in M<sup>-1</sup>),  $r$  is micrograms Ig bound per well,  $c$  is unbound Ig in M, and  $n$  is the limiting value for  $r$  as  $c$  becomes very large. The number of cells per well was counted as described (see Materials and Methods). To calculate the amount of specifically bound Ig, the observed values were corrected by subtraction of counts per minute bound in the presence of a large excess of unlabeled Ig; this blank was the same as that observed by incubating the labeled Ig with control fibroblasts or in empty wells (Fig. 3). Fig. 4 A shows the Scatchard plots derived from data obtained at different temperatures; the resulting equilibrium constants ( $K_a$  values) and number of binding sites per cell are summarized in Table II. The  $K_a$  was considerably higher at 4°C than at 37°C, indicating that the reaction is exothermal. The van't Hoff plot of this data (log  $K_a$  vs. 1/T), shown in Figure 4 B,

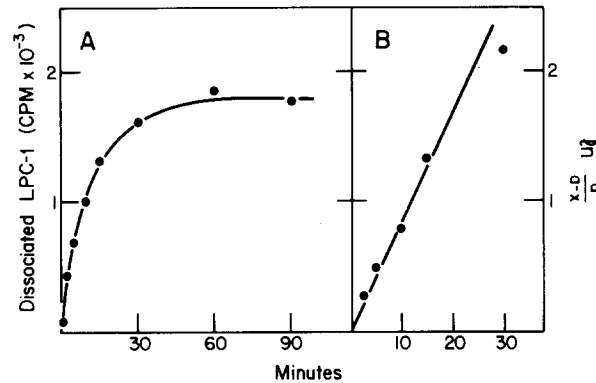


FIG. 2. Dissociation of bound LPC-1 myeloma protein from P388D<sub>1</sub> macrophages. Macrophages were incubated for 60 min with LPC-1 as in Fig. 1, washed rapidly, and then incubated in 0.5 ml of cold PBS containing 2 mg BSA/ml; samples were removed for assay at timed intervals. (A) Time-course of dissociation. (B) First-order plot of data in A; the value used for  $a$  was counts per minute released at completion of the reaction (average of counts per minute dissociation at 60- and 90-min time points in A);  $x$  was counts per minute dissociated at earlier time points.

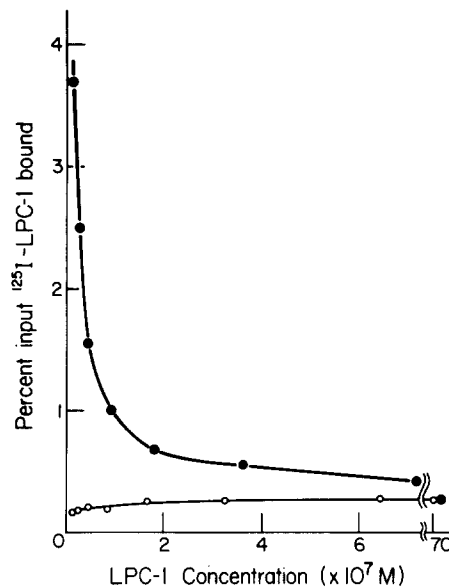


FIG. 3. Binding of <sup>125</sup>I-LPC-1 to cultured cells as a function of the molar concentration of LPC-1 (mol wt, 150,000 daltons). Cells were incubated at 4°C for 1 h with a constant amount of <sup>125</sup>I-LPC-1 (0.024 μg, 1.18 × 10<sup>5</sup> cpm) and the indicated amounts of unlabeled LPC-1. The data are not corrected for background binding: (●—●), 8 × 10<sup>5</sup> P388D<sub>1</sub> macrophages per well; (○—○), 1.2 × 10<sup>5</sup> C57BL/6J embryo fibroblasts per well.

suggests that binding varies with temperature, perhaps because of changes in the fluidity of the cell membrane below 20°C (12).

The binding of LPC-1 was independent of cellular energy metabolism: neither 2,4-dinitrophenol (10<sup>-5</sup> and 10<sup>-4</sup> M) nor fluoride (10<sup>-3</sup> and 10<sup>-2</sup> M) had an effect on binding. As others have found in examining the binding of aggregated Igs

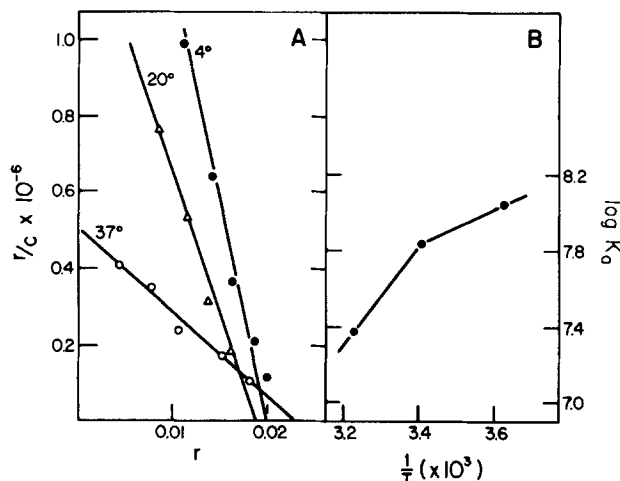


FIG. 4. The binding of LPC-1 to P388D<sub>1</sub> macrophages at different temperatures. Except for variations in temperatures the conditions were as in Fig. 3. Unbound Igs were removed in all instances with PBS at about 4°C,  $r$ , micrograms LPC-1 bound per well;  $c$ , unbound concentration of LPC-1 in moles per liter. (A), Variation of binding with free concentration of LPC-1 at 4°C, (●—●); 20°C, (△—△); and 37°C, (○—○). (B) Variation of equilibrium constants ( $K_a$ ) with absolute temperature ( $T$ ).

TABLE II  
*Equilibrium (Association) Constants and Number of Sites Per Cell for Binding of Intact LPC-1 and 5563 Myeloma Proteins and the Fab and Fc Fragments of LPC-1 to Macrophages of the P388D<sub>1</sub> Line*

Temperature	Protein	$K_a$	Sites per cell*
°C		( $M^{-1}$ )	
4	LPC-1	$1.1 \times 10^8$	100,000
24	LPC-1	$6.9 \times 10^7$	95,000
37	LPC-1	$2.2 \times 10^7$	110,000
37	5563	$2.9 \times 10^7$	100,000
37	LPC-1 Fab	—‡	—
37	LPC-1 Fc	$4.4 \times 10^6$	180,000§

Conditions for binding of LPC-1 and 5563 are described in Figs. 3 and 4. The Fab and Fc fragments were prepared as described. A constant input of  $^{125}I$ -Fab and  $^{125}I$ -Fc ( $0.05 \mu\text{g}$ ,  $2 \times 10^5$  cpm) was used; the amount of cold Fab or Fc was varied as described.

\* The number of binding sites per cell was obtained by multiplying the moles of bound Ig at saturation (abscissa intercept in Scatchard plot of Fig. 4) by Avogadro's number and dividing by the number of cells per well, determined as described.

‡ There was no detectable difference in the binding of the LPC-1 Fab fragment to macrophages and to mouse embryo fibroblasts.

§ The mol wt of the Fc fragment was assumed to be 50,000 daltons.

and Ig-coated erythrocytes (RBCs) (13, 14), the binding of monomeric LPC-1 was also independent of divalent cations: this protein in PBS-BSA was bound about as well in the presence or absence of EDTA (10 mM). Centrifugation of both the iodinated and unlabeled LPC-1 preparations for 3 h in a 50.1 SW rotor of a Beckman L65 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at

45,000 rpm (sufficient to pellet approximately 40% of the unlabeled protein) did not change the slope or intercept of the Scatchard plots. This observation and the volume of elution of the LPC-1 preparation from Sephadex G-200 strongly suggest that the bound protein was monomeric Ig, rather than a small subset of aggregated molecules.

**Binding of Fab and Fc Fragments.** The fragments prepared by papain digestion of LPC-1 were iodinated and tested for binding to P388D<sub>1</sub> cells. There was no detectable binding of the Fab fragment. As compared with intact molecules, the Fc fragments bound with slightly less affinity and to slightly more binding sites (Table II). The receptors on P388D<sub>1</sub> cells are clearly specific for the Fc domain of LPC-1.

**Trypsin Sensitivity of the Fc Receptor.** After treating P388D<sub>1</sub> macrophages with trypsin (1 mg/ml crystallized trypsin in PBS) for 15 min at 37°C the cells no longer bound LPC-1. However, when the trypsin-treated cells were incubated for 12 h in serum-free medium they regenerated 60% of their LPC-1-binding activity (Fig. 5). The receptors for LPC-1 on thioglycollate-stimulated macrophages were also sensitive to trypsin: their binding was reduced 96% by treating the cells with TPCK-treated trypsin (1 mg/ml) for 15 min at 37°C.

The effect of inhibitors of macromolecule synthesis on the regeneration of Fc receptors is shown in Fig. 6. After removing the receptors with trypsin as described above, the P388D<sub>1</sub> cells were incubated for 8 h in medium with 10%

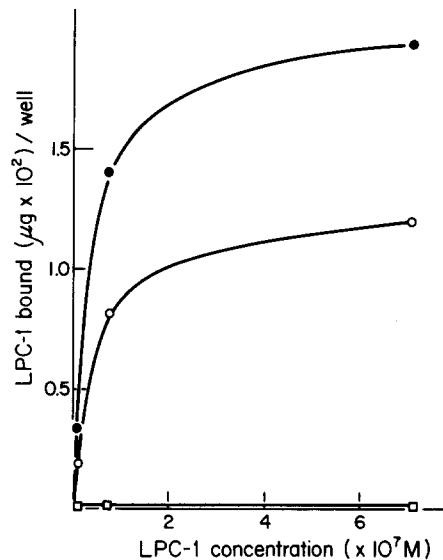


FIG. 5. Trypsin sensitivity of the IgG2a Fc receptor. After 24 h in MEM containing serum, P388D<sub>1</sub> macrophages ( $7 \times 10^6$ ) were washed and placed in serum-free MEM (with 0.05% lactalbumin hydrolysate) for 12 h before assay at 37°C for binding of LPC-1. After treatment with trypsin (1 mg/ml crystalline trypsin in PBS, 37°C, 15 min) the cells were washed twice with 0.5 ml portions of MEM containing 100  $\mu$ g/ml soybean trypsin inhibitor, and twice more with HBSS. (●—●), control, no trypsin treatment; (○—○), treated with trypsin before the 12-h incubation in serum-free medium before assay; and (□—□), treated with trypsin immediately before assay.

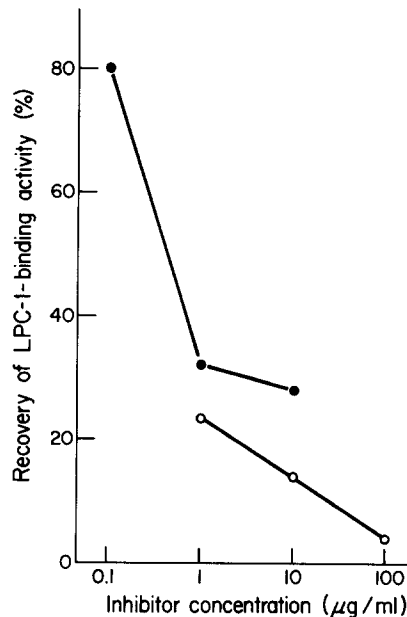


FIG. 6. Effects of actinomycin D and cycloheximide on the regeneration of Fc receptors after treatment of P388D<sub>1</sub> macrophages with trypsin. 12 h after plating, P388D<sub>1</sub> cells ( $7 \times 10^6$ ) were treated with trypsin as described in Fig. 5 and incubated for 8 h in MEM containing 10% heat-inactivated FCS and the indicated inhibitor. The cells were then washed and incubated in serum-free L-15 medium at 37°C for 30 min before assay with LPC-1 (1 μg/ml,  $5 \times 10^5$  cpm/μg) at 4°C. In the absence of inhibitors, trypsin-treated cells regenerated 36% of the LPC-1 binding-activity of untreated controls during the 8-h incubation period; i.e., 100% activity in the plot corresponds to 36% of the activity of untreated control cells. (●—●), actinomycin D; and (○—○), cycloheximide.

serum and cycloheximide or actinomycin D. Cycloheximide at 100 μg/ml totally inhibited recovery. At 1 and 10 μg/ml of actinomycin D regeneration of LPC-1-binding activity reached 30% of the level in control cells, which had been treated with trypsin and incubated in the absence of the inhibitors. Additional controls, in which cells were not treated with trypsin but incubated in presence of the inhibitors, retained at least 80% of the binding activity, relative to untreated P388D<sub>1</sub> cells. Cell loss due to trypsin or to inhibitor toxicity was negligible. The results are consistent with either an internal pool of receptors (or receptor subunits) whose assembly at the cell surface requires other protein synthesis, or a somewhat stable messenger RNA (for the Fc receptor), whose translation is blocked by cycloheximide. In either case the results establish that the surface Fc receptors are not simply adsorbed, but are produced by the cells that display them.

*Binding of other Myeloma Proteins and Comparison of Normal and Thioglycollate-stimulated Macrophages.* With the binding assay described, a number of other myeloma proteins were examined for cytophilic activity. In all cases, C57BL/6J embryo fibroblasts were also used as binding controls. Over a wide range of specific activities the binding of each labeled myeloma protein to fibroblasts remained constant at 0.2–0.5% of the input radioactivity.



Protein 5564, another IgG2a myeloma protein, bound to P388D<sub>1</sub> cells with nearly the same affinity constant as LPC-1. Titration of the binding of <sup>125</sup>I-LPC-1 was performed as in Fig. 3 but in the presence of unlabeled protein 5563, at two levels (5 and 25 μg/ml). The binding of the radioactive IgG2a protein was substantially inhibited by the unlabeled one, and the inhibition equilibrium constant,  $K_i$ , estimated for protein 5563 was about the same as the  $K_a$  value measured for the same protein in a direct binding titration, using <sup>125</sup>I-5563. From these results it appears that both of the IgG2a proteins were bound with the same affinity to the same sites on the macrophage surface. These two myeloma proteins have different isoelectric points: 5563 had four or five species centered around pH 5.5 and LPC-1 had a number of bands centered at pH 7.3. The differences probably reflect different amino acid sequences in the respective V<sub>H</sub> domains, while the uniformity of binding to macrophages is consistent with other evidence that these proteins are bound via C<sub>H</sub> domains (i.e., with LPC-1 the Fc fragment was bound and the Fab fragments were not).

As is shown in Figs. 7 A and 7 B, MPC-11 an IgG2b myeloma protein, was weakly cytophilic; at 4°C the  $K_a$  was  $7 \times 10^6 \text{ M}^{-1}$ , 20-fold less than the value for IgG2a. Because of the low association constant (Table III) the fraction bound was at most only fourfold over background; the binding of labeled MPC-11 to embryonic fibroblasts remained constant over the range of specific activities tested. Preliminary experiments suggest that there was mutual interference in the binding of LPC-1 and MPC-11, but whether these proteins bind to the same receptor or are mutually inhibitory because of steric hindrance is not clear. None of the other myelomas tested (MOPC-104E, an IgM; MOPC-21A, an IgG1; and T15, an IgA) inhibited the binding of LPC-1 even at a 70:1 excess (wt/wt).

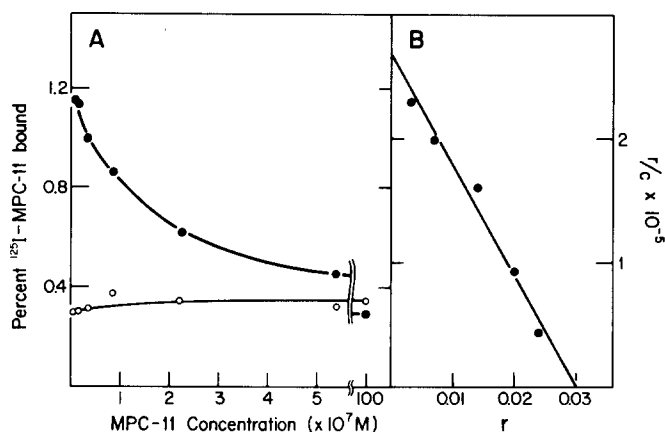


FIG. 7. Binding of <sup>125</sup>I-MPC-11 to cells as a function of MPC-11 concentration. 2 days after plating, cells were washed, incubated for 3 h in serum-free L-15 medium at 37°C, and then incubated at 4°C for 1 h with a constant amount of <sup>125</sup>I-MPC-11 (0.015 μg,  $1.09 \times 10^6$  cpm) and varying amounts of unlabeled MPC-11. The plates were washed in cold PBS and the bound protein released with trypsin. (A) Percent of <sup>125</sup>I-MPC-11 bound. (●—●), thioglycollate-stimulated macrophages,  $4 \times 10^5$  per well; and (○—○) C57BL/6J embryo fibroblasts,  $1.7 \times 10^5$  per well. (B) Data of A replotted to determine the equilibrium constant (slope) and number of binding-sites (abscissa intercept). For  $r$  and  $c$  see Fig. 4.

TABLE III  
*Equilibrium Constants and Number of Sites Per Cell on Normal and Thioglycollate-Stimulated Macrophages and on Cells of the P388D<sub>1</sub> Line for IgG2a and IgG2b Myeloma Proteins*

Protein	Type of macrophage	$K_a$ ( $M^{-1}$ )	Sites per cell
LPC-1 (IgG2a, $\kappa$ )	P388D <sub>1</sub>	$1.3 \times 10^6$	84,000
	Thioglycollate-stimulated	$1.3 \times 10^6$	440,000
	Normal	$1.4 \times 10^6$	110,000
MPC-11 (IgG2b, $\kappa$ )	P388D <sub>1</sub>	$7.5 \times 10^6$	110,000
	Thioglycollate-stimulated	$9.0 \times 10^6$	290,000
	Normal	$6.8 \times 10^6$	50,000

All assays were performed at 4°C. The number of cells per well at the termination of the experiment were: P388D<sub>1</sub> cells,  $1 \times 10^6$ ; thioglycollate macrophages,  $5 \times 10^5$ ; and normal macrophages,  $4 \times 10^5$ .

When iodine labeled, moreover, none of these myeloma proteins bound any more to macrophages than to mouse embryo fibroblasts.

The three types of macrophage tested did not differ in their affinity for the IgG2a proteins or in their affinity for MPC-11 (IgG2b). However, normal and P388D<sub>1</sub> macrophages had fewer sites per cell than the thioglycollate-stimulated cells for both LPC-1 and MPC-11.

### Discussion

The present study differs from previous work on mouse macrophage Fc receptors in that it examined the binding of monomeric Igs, rather than aggregated Igs (3) or Ig-coated RBCs [rosette formation (1, 13-17)]. Moreover, the use of a simple and rapid technique for washing the cells made it feasible to remove unbound Igs without significant loss of bound Igs through dissociation. This made it possible to measure rates of association and dissociation, equilibrium constants, and the number of binding sites per cell with confidence. Comparison of normal mouse peritoneal macrophages, thioglycollate-stimulated macrophages, and a macrophage cell line (P388D<sub>1</sub>) revealed that all three are similar in their binding of mouse Igs. Of the five Ig classes examined, IgA, IgM, and IgG1 were not bound significantly, IgG2a was bound with high affinity, and IgG2b was bound with a 20-fold lower affinity. These observations confirm results of Ralph et al. (3) who found that aggregated IgG2a and IgG2b were cytophilic. However, they conflict with the results of Cline et al. (17) who reported that only IgG2a, and to a lesser extent IgG1, can compete for the binding and phagocytosis of IgG-coated RBCs. Askenase and Hayden (16) suggest that in addition to receptors for IgG2a, there may be receptors for monomeric IgM on macrophages.

Berken and Benacerraf (18) found that the Fc fragment was responsible for the cytophilic properties of guinea pig IgG2, and others have since found that the Fc fragment of cytophilic Igs can inhibit binding and phagocytosis of Ig-coated RBCs (19) and binding of iodinated cytophilic Ig (20). Two observations in

this study established that the cell receptors for monomeric Ig were specific for Ig constant domains: (a) two IgG2a proteins (LPC-1 and 5563) that differed considerably in their isoelectric points, doubtlessly because of differences in their variable regions, were bound with the same affinity; and (b) with one of these proteins, LPC-1, the Fc fragment was bound and the Fab fragments were not.

The affinity of IgM for macrophage surface receptors is unclear. Ralph et al. (3) found no detectable binding of  $^{125}\text{I}$ -aggregated IgM; and in the present study macrophages were no more effective than embryonic mouse fibroblasts in binding unaggregated IgM. Cline et al. (17) found, in addition, that RBCs coated with IgM did not bind to mouse macrophages. However, Lay and Nussenzweig (13) have reported that sheep RBCs (SRBCs) coated with 19S antibodies to SRBC form rosettes with macrophages, and Askenase and Hayden (16) described partial inhibition of rosette formation by antisera to IgM. While there are some obvious contradictions, it remains possible that the binding of antigens induces a conformational change in the Fc domain of IgM proteins (21), and that this is required for significant binding of IgM molecules to macrophage receptors.

The present study clearly shows that the macrophage receptors for the Fc of IgG2a proteins are readily inactivated by trypsin. Other studies suggest, however, that Fc receptors for Igs may differ in trypsin sensitivity. Kossard and Nelson (15) reported that the receptors for cytophilic antibodies from early antisera to SRBCs were trypsin sensitive and that the receptors for cytophilic antibodies from late antisera were trypsin resistant. Similar findings were noted with the early and late antisera to oxazolone (16). Others have observed, with hyperimmune sera, that receptors for antibody-coated RBCs (13) and for antibody-antigen complexes (22) are entirely trypsin resistant. There may well be more than one type of Fc receptor on macrophages. The trypsin-sensitive receptors characterized in the present work probably correspond to those that bind cytophilic antibodies from early antisera; trypsin-resistant Fc receptors could have quite different properties.

Fc receptors on guinea pig macrophages have been shown to bind guinea pig IgG2, not IgG1 (18). The guinea pig cells bound IgG2 molecules with lower affinity and at more binding sites per cell (23, 24) than we observed here with mouse macrophages and mouse IgG2a molecules. (At 20°C, the values for stimulated guinea pig macrophages were  $1.5 \times 10^6 \text{ M}^{-1}$  and  $2.5 \times 10^6$  sites per cell vs.  $7 \times 10^7 \text{ M}^{-1}$  and  $4 \times 10^5$  sites per cell for the mouse system.) The similarities are great enough to suggest that there is considerable structural homology between guinea pig IgG2 and mouse IgG2a and IgG2b molecules. The differences could derive from the use of different procedures for measuring binding or from the use of homogeneous myeloma proteins in the mouse system and an IgG electrophoretic fraction in the guinea pig system (the latter could well include several classes as different as mouse IgG2a and IgG2b).

Each of the mouse macrophage types examined in the present study had the same affinity for monovalent mouse IgG2a ( $2 \times 10^7 \text{ M}^{-1}$  at 37°C); hence, with unbound IgG2a at a concentration of 7  $\mu\text{g/ml}$ , receptors on the cell surface would be half saturated. Since IgG2a is doubtlessly present in blood at a much higher concentration, the corresponding Fc receptors of macrophages are probably

saturated *in vivo*. From the number of Fc receptors per cell, the surface area of an unstimulated peritoneal macrophage ( $1,000 \mu\text{m}^2$ ) (R. Steinman, personal communication), and the cross-sectional area of a 7S Ig molecule, it can be calculated that about 1% of the cell surface is covered with IgG2a molecules when the corresponding receptors are saturated. Though the three cell types have the same affinity for IgG2a, there are fourfold more binding sites on thioglycollate-stimulated than on normal or P388D<sub>1</sub> macrophages. The difference is probably due to the greater size and surface area of the stimulated cells.

From the equilibrium constants and rate constants for dissociation, the bimolecular association rate constants for the binding of IgG2a to mouse macrophages are readily obtained. At 4°C, this value is  $0.9 \times 10^7 \text{ M}^{-1}\text{min}^{-1}$ . This constant and the measured dissociation rate constant at this temperature ( $0.08 \text{ min}^{-1}$ , Table I) are both similar to the kinetic constants for representative antibody-antigen reactions in solution. Dandliker and Levison (25), for instance, found the forward and reverse rate constants for the reaction of fluorescein-labeled ovalbumin with rabbit antibodies to ovalbumin at 1.5°C to be  $1.2 \times 10^7 \text{ M}^{-1}\text{min}^{-1}$  and  $0.06 \text{ min}^{-1}$ . Due to cooperative binding effects (24, 26), even more stable complexes are likely to result from the formation of aggregates that contain many antibody and many antigen molecules. In the macrophage membrane, however, bound monomeric IgG2a molecules are unlikely to form large, multimolecular aggregates with Fc receptors, since the equilibrium constants were independent of IgG2a concentration. (Even if the Fc receptors are fully mobile in the membrane, there are unlikely to be more than one or two binding sites per Fc domain of IgG2a.) Large aggregates could be formed in the cell membrane, however, if multivalent antigen particles were to cross-link IgG2a molecules before or after the latter are bound to Fc receptors.

While the IgG2a/Fc complex is relatively stable, the half time for dissociation is still fairly rapid (2.6 min at 37°C, Table I). This suggests that under physiological conditions the macrophage surface is in a dynamic steady state, binding and releasing IgG2a molecules rapidly enough to allow the cell's antigen-binding capabilities to mirror the specificities of free IgG2a molecules in the cell's immediate microenvironment. The binding properties of the macrophage Fc receptors seem well adapted to support the cell's capacity to bind, presumably via Ig molecules, an almost limitless variety of foreign antigens.

### Summary

The binding properties of surface receptors of immunoglobulins on mouse macrophages were studied with mouse myeloma proteins and normal peritoneal macrophages, thioglycollate-stimulated macrophages, and a macrophage cell line, P388D<sub>1</sub>. Primary cultures of mouse embryo fibroblasts served as controls. IgG2a proteins were bound strongly; IgG2b was bound weakly (one-twentieth as well as IgG2a); IgM, IgA, and IgG1 were not bound significantly. The number of binding sites per cell for IgG2a was  $4 \times 10^5$  for thioglycollate-stimulated cells and  $1 \times 10^5$  for normal and P388D<sub>1</sub> cells. Binding was exothermic: with decreasing temperature the equilibrium (association) constants increased and dissociation rate constants decreased (at 37°C the respective values were  $2 \times 10^7 \text{ M}^{-1}$  and  $0.26 \text{ min}^{-1}$ ; the latter value corresponds to a half time for dissociation of 2.6

min). From the rapidity of association and dissociation, it appears that the surface of the macrophage is in a dynamic equilibrium with IgG2a molecules in the cell's immediate microenvironment.

The receptors for IgG2a are clearly specific for determinants in the immunoglobulin constant domain: two IgG2a proteins with greatly different isoelectric points (determined by isoelectric focusing) were bound with the same affinity to the same receptors; moreover, the Fc fragment was bound and Fab fragments were not. The Fc receptors for IgG2a proteins were readily eliminated by exposing macrophages briefly to trypsin. The receptors were regenerated during subsequent cultivation in serum-free medium; regeneration was inhibited totally by cycloheximide and partially by actinomycin D.

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