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Immunology

Fc-receptor variants of a mouse macrophage cell line

(somatic cell genetics/complement receptor/antigen–antibody complexes/mouse IgG subclasses)

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ABSTRACT **Variants of the J774 mouse macrophage cell line that lack immunologically important membrane receptors were isolated. After mutagenesis, variants were selected in a metrizamide gradient that separated cells heavily rosetted with sheep erythrocytes (E) coated with rabbit anti-E IgG (EIgG) from poorly rosetted cells. Stable variants that exhibited altered binding were found with a frequency of $<10^{-7}$, and five clones were studied in detail. The variants failed to bind E opsonized with a monoclonal mouse IgG2b anti-E antibody but bound monomeric IgG2a normally when compared to the parental J774 line (K_a 4°C = $\approx 1 \times 10^8$ M^{-1}; $\approx 2 \times 10^5$ sites per cell). This demonstrates the independence of the receptor for mouse IgG2b complexes (FcRII) from the trypsin-sensitive receptor for mouse IgG2a monomer (FcRI). The variants bound an average of 10–15 EIgG per cell, compared to >20 per cell for J774. After trypsinization, three variants bound only three to five EIgG per cell; the J774 line was not affected by this treatment. Monomeric IgG2a could inhibit the binding of soluble rabbit IgG–antigen complexes to the variants but not to the parent line. Finally, E coated with IgM and complement (EIgMC) were bound poorly by all the variants, relative to the J774 parent. These results show that rabbit IgG complexes are bound by both FcRI and FcRII on mouse macrophages. The impairment of EIgMC rosetting in the variants suggests that the C3b receptor and FcRII are related.**

The role of macrophages in host defense is partially governed by the presence of immunological receptors on their plasma membrane. These receptors lead to the binding of both the C3b component of complement and the Fc domain of IgG and specify the phagocytosis of particles (1) or the extent of cell-mediated cytotoxicity (2, 3). Some controversy exists about the number and types of Fc receptors on mouse macrophages (4–9). Native macrophages and cell lines, however, display an Fc receptor (FcRII) that binds antigen–antibody complexes and aggregated mouse IgG2b and is resistant to trypsin (5–7) as well as a trypsin-sensitive FcRI receptor that binds monomeric IgG2a with high affinity (8).

In order to characterize the Fc receptors further, we thought it would be useful to isolate variants with altered IgG binding. For this purpose the J774 cell line, which displays many of the characteristics of mouse peritoneal macrophages (10), was mutagenized and variant classes were isolated by a new density gradient technique.

MATERIALS AND METHODS

Cell Culture. The cell line J774 obtained from Paul Edelson was recloned by limiting dilution. The clone used for this work was designated G8 and was grown (5) in α modified minimum Eagle's medium with 10% heat-activated fetal calf serum (Flow Laboratories, Rockville, MD).

Preparation of Erythrocytes. Erythrocytes (E) were coated with either rabbit anti-E IgG (lot 90656, Cordis Laboratories, Miami, FL) at a nonhemagglutinating titer (EIgG) or with anti-E IgM (lot 90906, Cordis Laboratories) first absorbed with *Staphylococcus aureus* (11) according to Bianco *et al.* (12) (EIgM). Serum from Rockefeller NCS mice was used as the source of complement.

Mutagenesis. Cultures in exponential growth were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Aldrich) at a concentration of 1 μg/ml in Hanks' solution for 1 hr or with ICR-170 (0.3 μg/ml) for 16 hr in medium containing 10% fetal calf serum. The ICR-170 was kindly given to us by H. J. Creech (Chemotherapy Laboratory, Institute for Cancer Research, Philadelphia). Cell survival in populations treated with either mutagen under these conditions was 10%. The mutagenized cells were seeded on 100-mm dishes for 5 days and then reestablished in suspension culture.

Selection Procedure. Cells in late logarithmic phase 2–4 weeks after mutagenesis were resuspended in 2 ml of a 1:1 mixture of Hanks' solution and 0.25 M glucose (H/G) at a concentration of 10^7 cells per ml, and 2 ml of 5% EIgG in H/G was added. The suspension was mixed gently on a rotating wheel (15 rpm) for 15 min at 37°C and layered over 3 ml of isotonic metrizamide (Accurate Chemical and Scientific Corp., Hicksville, NY) (refractive index = 1.3572; ρ = 1.089 g/cm^3) prepared as described (13). After centrifugation at 2000 × *g* for 15 min, free EIgG and most rosetted cells were found in the pellet. The metrizamide–H/G interface, which contained poorly rosetted cells and some giant cells, was removed by pipette. The cells were washed by centrifugation and seeded in 100-mm dishes in 50% J774-conditioned medium. Alternatively, cells from the interface were maintained in suspension and reselected 4–16 hr later.

The cells from the metrizamide–H/G interface were grown until the colonies contained 20–100 cells. The dishes were treated with 0.25% 1:300 trypsin (GIBCO) in phosphate-buffered saline without divalent cations for 15 min at 37°C, washed with medium containing 10% serum, and incubated with 4 ml of 1.25% EIgG in H/G for 20 min at 37°C. After gentle washing to remove nonadherent EIgG, colonies with altered phenotype were simultaneously dislodged with a sterile plastic micropipette tip and removed in 20 μl of medium. The cells were grown in 60-mm dishes in 50% J774-conditioned medium and then cloned by limiting dilution.

Proteins and Immunoglobulins. Rabbit anti-dinitrophenyl-IgG (DNP-IgG), the F(ab')$_2$ fragment of rabbit IgG, $DNP_{10.9}$-bovine serum albumin, and the mouse IgG2a myeloma protein LPC-1 were prepared and iodinated with ^{125}I as described (5, 8, 14). The monoclonal anti-sheep erythrocyte IgG2b, U88, was given to us by B. Diamond and M. Scharff (Albert Einstein Medical School).

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Abbreviations: E, sheep erythrocytes; EIgG, E coated with rabbit IgG; EIgG2b, E coated with monoclonal mouse IgG2b; EIgM, E coated with rabbit IgM; EIgMC, E coated with rabbit IgM and the C3b component of complement; H/G, 1:1 mixture of Hanks' solution and 0.25 M glucose; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DNP, dinitrophenyl.

Binding Assays. Binding assays were performed on adherent cells in 16-mm wells (Linbro FB 16-24 TC) as described (5, 8) except that, after washing, the cells were removed from the dish by swabbing the wells with Q-tips, which were then assayed for radioactivity. Rabbit anti-DNP-IgG–$DNP_{10.9}$-albumin complexes were formed at a 5:1 (wt/wt) ratio which gave optimal cell binding. The binding of complexes or IgG2a to L cells was linear and these values were used to correct for nonspecific binding. Rabbit anti-DNP-$F(ab')_2$–$DNP_{10.9}$-albumin complexes bound equally to L cells and cells of the J774 line.

EIgG and C3b-Coated EIgM (EIgMC) Binding. Cells grown overnight on 13-mm glass coverslips were incubated for 20 min at 37°C with 1 ml of 0.5% EIgG or EIgMC suspended in H/G and then washed free of nonadherent cells by dipping in phosphate-buffered saline. In some experiments cells were trypsinized [1 mg of crystalline trypsin (Sigma) per ml, 15 min, 37°C] prior to rosetting. The cells were fixed in 2.5% glutaraldehyde in phosphate-buffered saline, and at least 200 cells were scored for EIgG and EIgMC binding.

Latex Phagocytosis. Washed polyvinyltoluene latex particles (2.02 μm; Dow Diagnostics) were suspended in 5% serum medium and 5×10^7 particles were added to cells for 1 hr at 37°C.

Lysozyme Secretion. Lysozyme was assayed as described (15) with rat lysozyme as a standard.

Scanning Electron Microscopy. Cultures fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer, pH 7.3, were dehydrated in alcohol, transferred to amyl acetate, and critical point dried (Sorvall C.P.D. System) in liquid CO_2. The cells were coated with gold (Edwards 5150 sputter coater) and viewed with an ETEC autoscan.

RESULTS

Selection of Variants. The J774 macrophage cell line (10) was chosen because of its rapid cell cycle and high cloning efficiency. The cells grow either in suspension or as adherent cells, and they form dense rosettes with EIgG or EIgMC but not with E and EIgM—a spectrum of erythrocyte binding identical to that of native macrophages.

We selected variants by isopycnic centrifugation of cells rosetted with EIgG on a shelf gradient of metrizamide. By this procedure, heavily rosetted cells were separated from nonrosetted or lightly rosetted variants, which collect at the interface of the metrizamide. The density of the metrizamide solution (1.089 g/cm^3) was just sufficient to float J774 cells. A mock selection, in which E instead of EIgG were incubated with the J774 cells, confirmed that J774 cells remained at the interface between metrizamide and saline after centrifugation. When J774 cells were incubated with EIgG, about 0.1% of the colony-forming cells in the initial population were recovered at

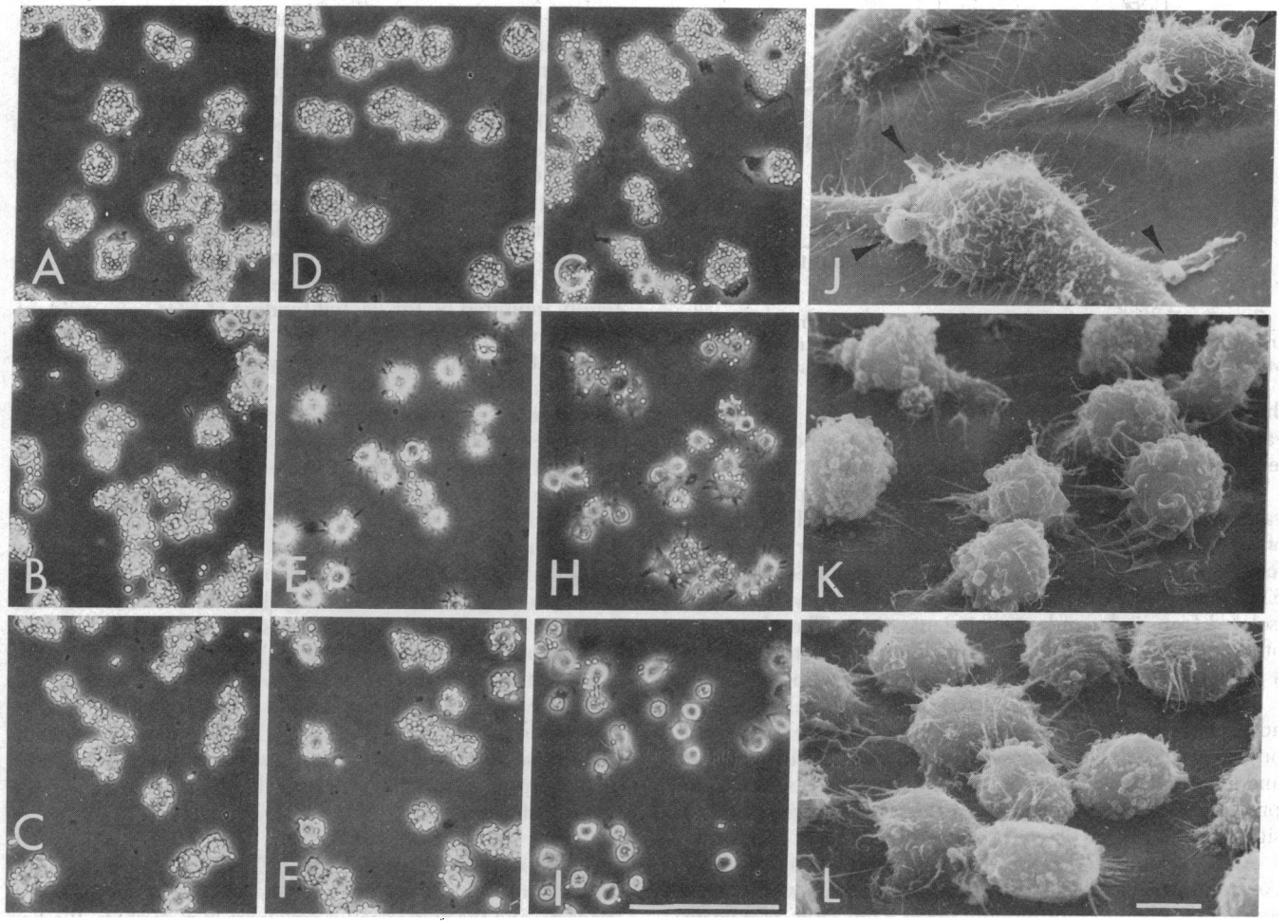

FIG. 1. Rosetting of EIgG and EIgMC to J774, ICR 4.4, and MNNG 1.2 cells. (*A–C*) EIgG incubated with J774 (*A*), ICR 4.4 (*B*), and MNNG 1.2 (*C*). (*D–F*) EIgG incubated with cells first trypsinized with 1 mg of trypsin per ml for 15 min at 37°C: J774 (*D*), ICR 4.4 (*E*), and MNNG 1.2 (*F*). (*G–I*) EIgMC incubated with J774 (*G*), ICR 4.4 (*H*), and MNNG 1.2 (*I*). Scanning electron microscopy of J774 (*J*), ICR 4.4 (*K*), and MNNG 1.2 (*L*). Arrowheads in *J* locate membrane ruffles seen on parental lines and absent on variants. Viability of cells in these cultures was >95% and there were many mitotic figures. Bars: in *I*, 100 μm; in *L*, 10 μm.

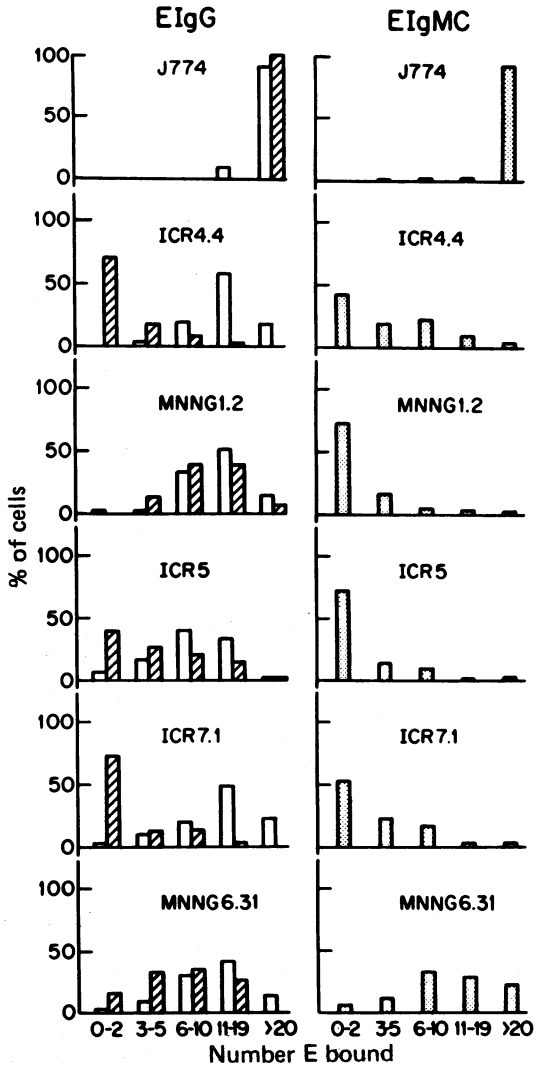

FIG. 2. Fc receptor and C3b receptor-mediated binding of EIgG and EIgMC to variant lines. Cells after trypsin treatment are shown with hatched bars. Trypsin treatment was as in Fig. 1.

the metrizamide-H/G interface. To facilitate selection of variants present at low frequencies, we often repeated the selection procedure after a 4- to 16-hr interval.

Virtually 100% of J774 cells express Fc receptors and no variants have ever been found in nonmutagenized populations of J774 cells. After mutagenesis by either MNNG or the acridine half-mustard ICR-170 (16), cells exhibiting altered binding of EIgG were found with a frequency of $<10^{-7}$. The variants were isolated from independent mutagenizations. The phenotype of the isolated clones and their subclones has been stable over a period of at least 6 months (>300 cell generations).

Mixing experiments in which 1×10^6 ICR 7.1 cells were added to 2×10^7 J774 revealed that >80% of the variant colony-forming cells were recovered at the metrizamide interface and indicated that the method did not introduce discriminatory pressure. This was the case even though the variants bound significant numbers of erythrocytes.

Analysis of EIgG Binding. The J774 parent cells were totally covered with EIgG (>25) whereas cells from clones MNNG 1.2 and ICR 4.4 bound substantially fewer EIgG (Fig. 1). The distribution of binding to individual cells and the average number of EIgG bound per cell are shown in Fig. 2 and Table 1. The variants bound an average of 10–15 EIgG per cell. The presence of a trypsin-sensitive receptor on the variants was next examined

Table 1. Binding of EIgG and EIgMC to J774 and variant lines

Cell tested	Average number of E bound* EIgG	EIgG on trypsinized macrophages	EIgMC
J774	24	25	24
ICR 4.4	14	3.0	5.6
MNNG 1.2	15	11	2.9
ICR 5	10	5.3	3.0
ICR 7.1	14	2.6	4.0
MNNG 6.31	13	7.7	13

* The values were calculated from data in Fig. 2 by multiplying the percentage of cells in each category by the average number of E bound in that category, summing the total, and dividing by 100. The number of E in the >20 category was set at 25.

because trypsinization removes the binding of monomeric IgG2a on macrophages and cell lines (8) but does not affect the binding of EIgG to the parent line J774. Surprisingly, three variants (ICR 5, ICR 4.4, and ICR 7.1) showed markedly reduced binding of EIgG after trypsinization.

Binding of Immune Complexes. A more quantitative estimate of binding was accomplished with radiolabeled immune complexes prepared with rabbit antibody (Fig. 3). At saturation, all the variants bound less than 20% of the amount bound by the J774 parent line (0.05 μg/3 $\times$ 10^5 cells). Because the binding of EIgG to some of the variants was trypsin-sensitive, and the FcRI that binds IgG2a was likewise trypsin-sensitive, we added IgG2a to the incubation mixtures to compete for the binding of the soluble rabbit immune complexes. The amount of IgG2a used (0.05 mg/ml) was about 30 times the K_m for IgG2a at 4°C and largely inhibited the binding of complexes by the variants (Fig. 3). Only slight inhibition occurred with the parent line and none with L cells which lack Fc receptors. These results suggest that soluble rabbit immune complexes bind to both trypsin-sensitive and trypsin-resistant Fc receptors. The behavior of MNNG 1.2 was anomalous, because binding of complexes was totally inhibited by IgG2a, but binding of EIgG was resistant to trypsinization.

Rosetting with EIgG2b. The trypsin-resistant Fc receptor has been reported to bind aggregated mouse IgG2b. We therefore tested the binding of E coated with the monoclonal IgG2b (EIgG2b) anti-sheep erythrocyte antibody U88, kindly supplied by B. Diamond and M. Scharff. E coated with a less than hemagglutinating concentration of this monoclonal IgG2b bound to J774 cells, and the formation of rosettes was not inhibited by treatment of the J774 cells with trypsin at 2 mg/ml for 30 min at 37°C. The variants bound few or no EIgG2b, as shown in Fig. 4, demonstrating that these clones lack the trypsin-resistant receptor that binds IgG2b.

Binding of Monomeric IgG2a. The presence of the trypsin-sensitive FcRI on the variants was examined by using the monomeric mouse IgG2a myeloma protein LPC-1. Scatchard analysis of the binding data showed that the variant lines bound the mouse IgG2a myeloma protein with slightly higher affinity to a comparable number of sites (Table 2), confirming that the variants have the FcRI for IgG2a.

Other Cellular Characteristics. The presence of the C3b receptor was assayed with EIgMC. EIgM did not bind to either variant or parent lines. Nearly 100% of the parental clone bound EIgMC, and the only negative cells were in metaphase. Binding of EIgMC to all the variant lines was decreased (Figs. 1 and 2). This result was also unexpected because there was no obvious reason why a selection against EIgG-binding cells should select against EIgMC rosetting.

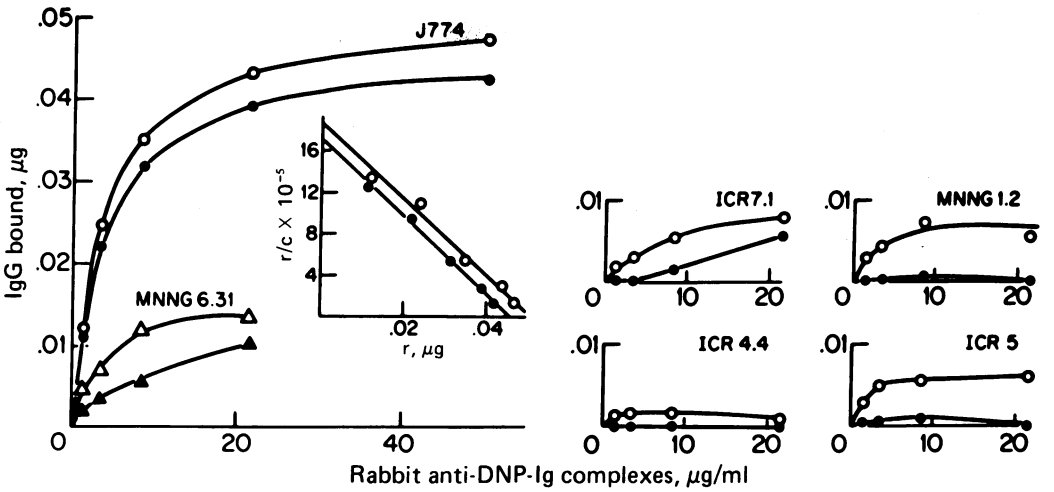

FIG. 3. Binding of rabbit antibody–antigen complexes to variants and competition of binding by mouse IgG2a. ○ and △, Binding of rabbit anti-DNP–$DNP_{10.9}$-albumin complexes; ● and ▲, binding of the same complexes in the presence of the mouse IgG2a myeloma protein LPC-1 (0.05 mg/ml). The wells were seeded with 3×10^5 cells 4 hr before the experiment.

Mouse peritoneal macrophages and the J774 cells phagocytose latex particles and secrete lysozyme. Table 2 shows that the variant clones were normal in both respects. All the variants, with the exception of MNNG 6.31, did not adhere to glass or plastic surfaces as tenaciously as did the parent line, and the variants, with the exception of ICR 4.4, were detached from the surface of plastic, but not glass, after trypsinization. Scanning electron microscopy (Fig. 1) revealed that the parent line was well spread, showed polarity, and exhibited membrane ruffling and ridges characteristic of macrophages. These features were not seen in the variants, which were less well spread and whose surface topography was less regular, with many blunt processes and blebs.

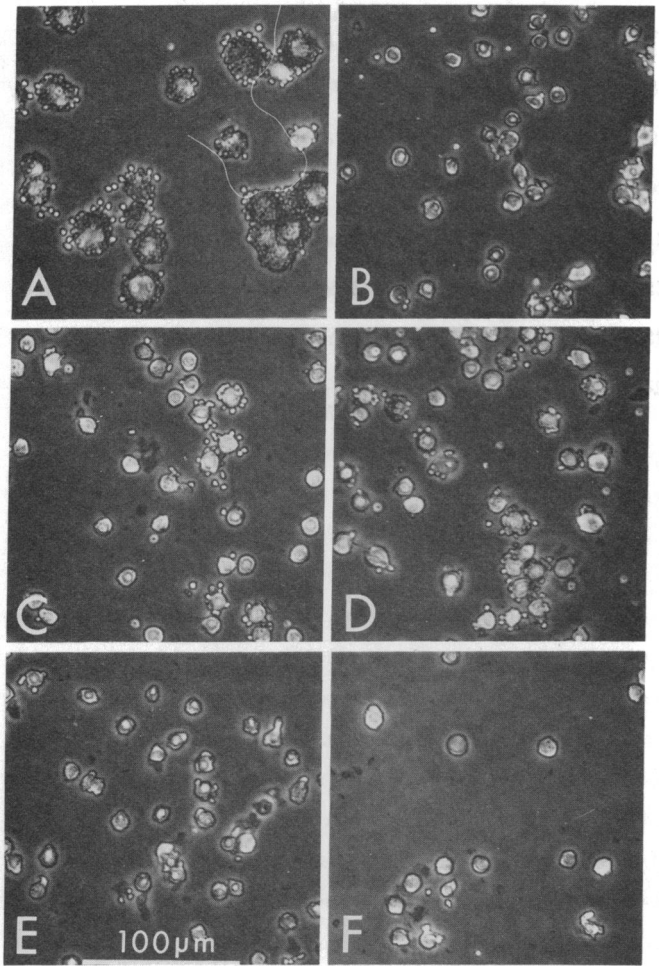

FIG. 4. Rosetting of J774 and variants by EIgG2b. (*A*) J774, (*B*) ICR 4.4, (*C*) MNNG 1.2, (*D*) ICR 5, (*E*) ICR 7.1, and (*F*) MNNG 6.31.

DISCUSSION

Variant clones with substantially decreased binding of EIgG were obtained from a macrophage cell line, J774, after mutagenesis and selection based on physical separation of rosetted from nonrosetted cells. The variants did not form rosettes with EIgG2b; EIgG2b bound to the parent J774 clone via a trypsin-resistant Fc receptor (FcRII) as shown by Diamond *et al.* (9). Although the variant clones showed a dramatic inability to bind EIgG2b, they did bind monomeric mouse IgG2a normally. The IgG2a subclass is bound at a trypsin-sensitive site in the parental clone (FcRI). We suggest that rabbit antibody–antigen complexes have affinity for both Fc receptors, because the binding of complexes to variants lacking the FcRII can be inhibited by addition of monomeric IgG2a. The principal inconsistency in this model is that the formation of EIgG rosettes on two of the variants, MNNG 1.2 and MNNG 6.31, was not affected by trypsinization. Some possible explanations are that the FcRI of these two variants is resistant to trypsin treatment, or that the FcRII is masked and exposed after trypsinization.

Table 2. Characteristics of J774 and variant clones

Cell line	Latex ingestion*	Lysozyme secretion†	IgG2a binding: Affinity, $M^{-1} \times 10^{-8}$	IgG2a binding: Sites per cell $\times 10^{-5}$
J774	10	1.1	0.9	3.1
ICR 4.4	11	1.6	1.8	2.1
MNNG 1.2	8	2.4	1.4	2.9
ICR 5	9	0.8	1.8	1.5
ICR 7.1	7	1.1	1.9	1.9
MNNG 6.31	7	0.7	1.1	4.3
L cell	0	0		

* Particles per cell.

† Lysozyme secreted in a 24-hr period, shown as μg of lysozyme per mg of cell protein.

An unexpected result of these experiments was the decrease in binding of EIgMC by all the variants lacking FcRII. Because Fc receptors and C3b receptors appear at different times during macrophage maturation (17), and also differ in trypsin sensitivity (18), these two receptors are probably not the same. They might, however, share common polypeptides or be affected by a common processing, insertion, or glycosylation defect. Glycosylation defects that block insertion of the Thy-1 protein and also affect glycosylation of other cell surface components have been reported by Trowbridge *et al.* (19). In a study of the abnormal glycosylation of immunoglobulin heavy chain in a myeloma mutant, Weitzman *et al.* (20) found abnormal glycosylation of the H-2D but not the H-2K product of the mouse major histocompatibility locus. Because the morphologic and adherence properties of the variants were so different from those of J774, the defect(s) may involve general membrane processing steps.

Muschel *et al.* (21) devised a "Trojan horse" selection scheme in which EIgG loaded with the toxic drug tubercidin were fed to J774 cells to isolate variants deficient in phagocytosis of EIgG. These variants were isolated at a frequency of 1 per 1.7×10^4 MNNG mutagenized cells, a much higher frequency than that reported here for FcRII variants. Although the "Trojan horse" selection could produce variants that were not killed because of lack of EIgG binding, such variants were not found, which may reflect the difficulty of isolating variants present at a very low frequency in a much larger population of poorly phagocytic cells.

There have been several reports on the isolation of macrophage Fc receptors, but there is a lack of general agreement on their structure. Loube *et al.* (22) reported three polypeptides of M_r 57,000, 28,000, and 24,000; Bourgois *et al.* (23) reported a peptide of M_r 120,000 present on macrophages, lymphocytes, and fibroblasts; and Cunningham-Rundles *et al.* (24) reported a peptide of M_r 60,000 from human mononuclear cells. FcRII variant clones described here will be useful for the investigation of the function of the Fc and complement receptors and in providing favorable material for the isolation of the FcRI that binds IgG2a.

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