MACROPHAGE ACTIVATION SELECTIVELY ENHANCES EXPRESSION OF Fc RECEPTORS FOR IgG2a*

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Mouse macrophages (Mϕ) express distinct receptors for the Fc domain of IgG (FcR), a trypsin-resistant FcR for IgG2b/IgG1, a trypsin-sensitive receptor for IgG2a, and a third FcR for aggregated IgG3 (1, 2). In Mϕ, the FcR can mediate antibody-dependent endocytosis and cytolysis, trigger the respiratory burst (RB), and control secretion of reactive intermediates of oxygen, neutral proteinases, and prostaglandins (3–5). Mouse peritoneal Mϕ obtained after intraperitoneal infection with live bacillus-Calmette-Guérin (BCG-PM) express characteristically altered properties compared with Mϕ elicited by a sterile inflammatory agent, thioglycollate broth (TPM), or "resident" Mϕ from untreated animals (RPM) (6). Only BCG-PM release large amounts of H_2O_2 after surface stimulation, kill organisms and target cells more effectively, and are considered to be activated (7). Mϕ activated with BCG and other agents express enhanced Ia antigen (Ag), but reduced levels of a Mϕ-specific Ag F4/80 and of lectin-like receptors that mediate endocytosis of mannose-terminal glycoproteins (MFR) (6, 8). BCG-PM also bind decreased levels of 2.4G2 a monoclonal rat anti-mouse FcR antibody (Ab) that blocks rosetting of IgG2b- but not IgG2a-opsonized sheep erythrocytes (9).

We show that BCG infection enhances expression of the Mϕ FcR for IgG2a, but not IgG2b. Isotype specificity of FcR can be reversed in nonactivated Mϕ by BCG-induced lymphokines, and IgG2a immune complexes are more effective triggers of the RB in activated Mϕ.

Materials and Methods

Media and Reagents. Iscove's modified medium (IM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and other reagents were obtained as described (6).

Antibodies. Ab 2.4G2 was used as a pure protein or Fab fragment (a gift from Dr. J. Unkeless, The Rockefeller University, New York). Monoclonal anti-sheep erythrocyte (E) Ab U88 (IgG2b) and UM-2 (IgG2a) were from Dr. B. Diamond (Albert Einstein College of Medicine, Bronx, NY) (10). Monoclonal anti-dinitrophenyl (DNP) Ab K3 (IgG2a) and K1 (IgG2b) were from Dr. B. Askonas, Mill Hill, London, England and used as ascites or a protein purified by Dr. R. Dwek, University of Oxford.

Peritoneal Cells. RPM, TPM, and BCG-PM (6) were plated in 24- or 96-well tissue culture trays (Linbro Chemical Co., Flow Laboratories, Irvine, Scotland), or on glass coverslips at 1×10^5 Mϕ/well or 5×10^5 Mϕ/well, respectively.

Lymphokine. Lymphokine (8) was used at 5% vol/vol.

Binding of IgG. Cells were cultured for 4 h–2 d in IM + 5% FBS, washed in PBS, and cultured for 2 h in serum-free IM containing 0.05% lactalbumin hydrolysate for 2 h to allow dissociation or endocytosis of membrane-bound Ig. The medium was replaced by PBS (11)

with a mixture of cold and trace-labeled IgG2aαDNP (2.5 × 10^4 cpm/ng) and IgG2bαDNP (3 × 10^3 cpm/ng). A constant input of ^{125}I protein was used in all wells. After 30–90 min incubation at 4°C, unbound Ig was rapidly removed by washing three times with ice-cold PBS. To determine surface-bound Ig, cells were dissolved in 200 μl of 1 N NaOH and cell-associated radioactivity was measured. All assays were in duplicate, and variation from the mean usually did not exceed 10%. To determine whether ligand bound was at saturation, cells were incubated for 60 min with a constant amount of trace-labeled protein, excess cold protein was added, and radioactivity was measured after 5, 10, 15, 30, and 60 min. Background levels were reached by 30 min.

Binding and Phagocytosis of EIgG. Mϕ on coverslips were placed in 0.5 ml IM in wells. 0.1 ml E coated with subagglutinating concentrations of monoclonal mouse anti-E IgG2a or IgG2b were added. After 60 min at 4°C or 37°C, nonattached E were removed, Mϕ fixed in 0.5% glutaraldehyde in PBS, and attached E counted by phase contrast microscopy (12).

Superoxide (O_2^-) release. Linbro wells were coated with gelatin-DNP (13) by overnight incubation at 45°C and incubated with saturating concentrations of IgG2a or IgG2bαDNP Ab for 30 min at 37°C, washed twice, and 0.45 ml of reaction mixture was added (14). 5 × 10^5 Mϕ purified on a 17% metrizamide gradient were added (50 μl). Lymphokine-activated Mϕ were assayed after 24 h treatment in T-25 tissue culture flasks (Falcon Labware, Oxnard, CA) coated with 1% gelatin to prevent adherence. Results were expressed as nmol O_2^-/mg cell protein/60 min.

Results

Binding of Monomeric and Complexed IgG Subclasses. Adherent BCG-PM, TPM, and RPM, >90% pure, were incubated at 4°C with EIgG of different subclasses. Table I shows that binding of EIgG2a was enhanced on both BCG-PM and TPM, compared with RPM (Fig. 1 a,c) (twofold increase in EIgG2a bound/100 Mϕ and almost all Mϕ labeled), whereas binding of EIgG2b was selectively decreased on BCG-PM. Ingestion of EIgG2a by BCG-PM was also increased, unlike uptake of EIgG2b, and experiments with opsonised ^{51}Cr-labeled E gave similar results (not shown).

Unkeless and Eisen (11) found high affinity binding of monomeric IgG2a and IgG2b myeloma proteins to RPM, TPM, and the Mϕ cell line P388D1 and showed that the FcR for IgG2a was sensitive to trypsin. To study binding of monomeric IgG, Mϕ were incubated at 4°C with ^{125}I-labeled, uncomplexed monoclonal anti-DNP Ab of each subclass in the presence or absence of unlabeled Ig. The number of sites per cell was obtained by Scatchard analysis and specific binding at saturation. Table I shows that assays of monomer binding and rosetting were in good agreement. BCG-PM showed a 70% increase in the number of IgG2a sites and a 30% decrease in IgG2b sites compared with RPM, whereas both receptors were increased on TPM. There

TABLE I

Binding of Monomeric and Complexed IgG Subclasses to Murine Peritoneal Mϕ

| Targets | EIgG2a | | ^{125}I-IgG2a | | EIgG2b | | ^{125}I-IgG2b | |
	Percent Mϕ ro-setted	RBC/100 Mϕ	Sites/Mϕ	Ka (M)$^{-1}$	Percent Mϕ ro-setted	RBC/100 Mϕ	Sites/Mϕ	Ka (M)$^{-1}$
BCG-PM	95	665	110,000	2.2 × 10^8	68	360	65,000	4.0 × 10^7
RPM	65	320	72,000	4.1 × 10^8	80	480	90,000	8.0 × 10^7
TPM	86	602	120,000	3.1 × 10^8	94	720	200,000	1.2 × 10^8

* Mϕ were adhered to glass coverslips in 24- or 96-well trays for 2 h before assays of binding of complexed or monomeric IgG subclasses, respectively. Controls without EIgG showed <1% binding. Binding to live Mϕ of monomeric ^{125}I-labeled monoclonal mouse anti-DNP ab was measured at 4°C for 60 min. Binding to empty wells, L cell fibroblasts, or Mϕ in the presence of 100-fold excess of unlabeled myeloma protein was <0.5% of input. The number of sites determined by Scatchard analysis and by binding at saturation were within 5% of values shown. Results shown are representative of at least three experiments.
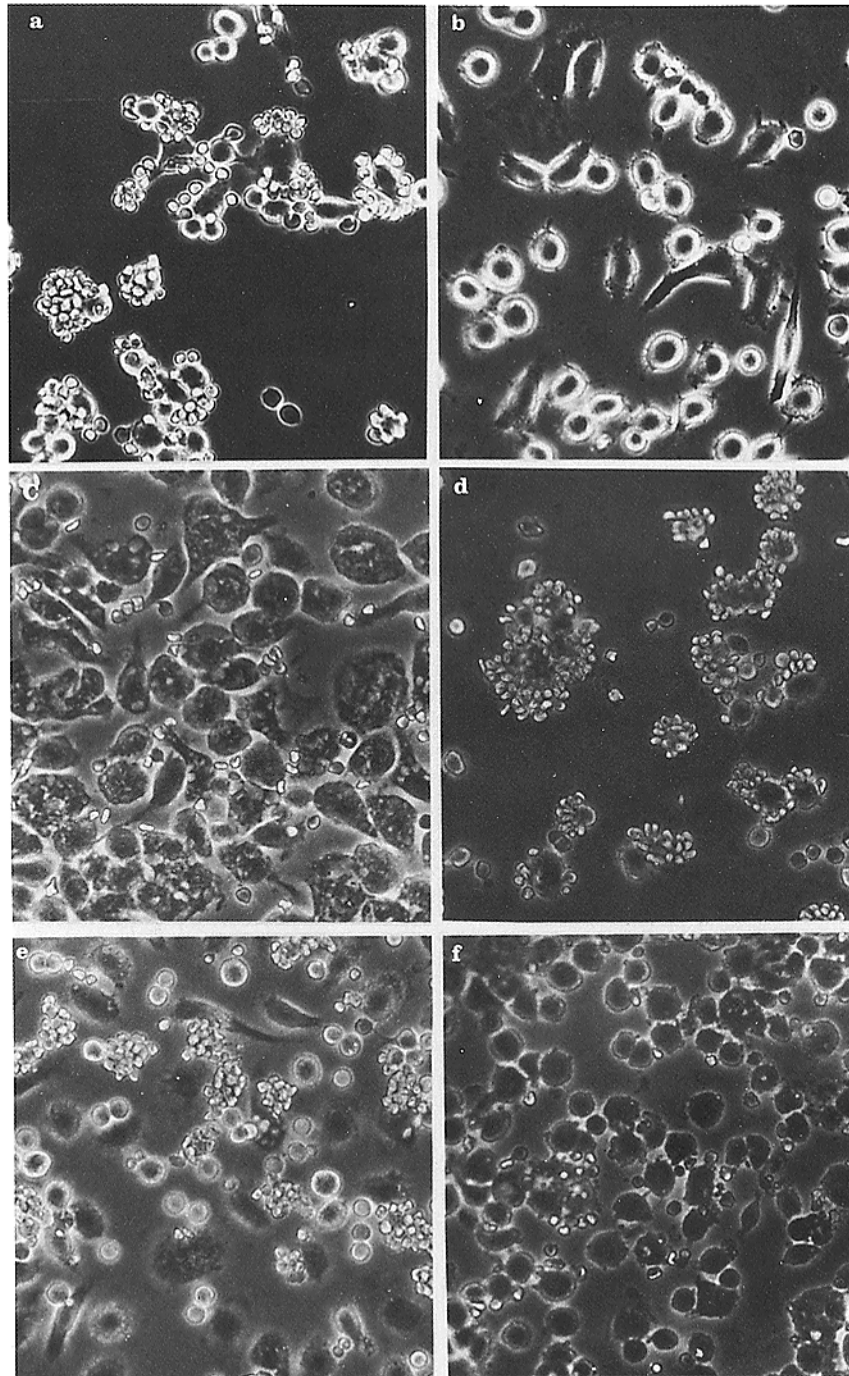
Fig. 1. Rosetting assays for Mφ FcR. (a) BCG-PM bind EIgG2a. (b) Modulation of IgG2a FcR. BCG-PM plated for 2 h on DNP-gelatin anti-DNP (IgG2a) complexes bind 30 E/100 Mφ compared with 540 E/100 Mφ plated on DNP-gelatin alone (a). (c–f) Lymphokine (LK) selectively enhances expression of FcR for IgG2a. RPM were treated with 5% vol/vol LK for 24 h before assay. (c) RPM, EIgG2a; (d) RPM + LK, EIgG2a; (e) RPM, EIgG2b; (f) RPM + LK, EIgG2b. Monomer binding assays, in parallel, gave the following results: IgG2a − RPM, 76,000 sites/cell, Ka 4.0 × 10^8 M^{-1}; RPM + LK, 125,000 sites/cell, Ka 2.8 × 10^8 M^{-1}; IgG2b − RPM 90,000 sites/cell, RPM + LK, 78,000 sites/cell, Ka not done. Experiments shown are representative of at least three independent assays.

TABLE II

Effect of Immobilized Immune Complexes on Fc-mediated O_2^- Production and on FcR Modulation

Addition to DNP-gelatin coated plate	Superoxide release				Modulation	
	nmol O_2^-/mg protein/60 min produced by				Percent BCG-PM with rosettes	
	BCG-PM	RPM	RPM + Lymphokine	TPM	EIgG2a	EIgG2b
None	160	12	40	26	92	60
PMA (20 ng/well)	316	67	120	98	ND*	ND
IgG2a anti-DNP	310	48	118	35	6	52
IgG2b anti-DNP	225	64	55	42	54	12

Assays of O_2^- release (>80% inhibited by superoxide dismutase) were performed in duplicate. Results representative of five independent experiments; variation from mean <10%. To modulate FcR, BCG-PM were cultivated on fixed immune complexes and binding of EIgG2a and EIgG2b measured at 4°C for 45 min.

* Not done.

was little change in affinity of each FcR on different Mϕ. The volume of elution of both IgG subclasses on Sephadex G-200 indicated that the proteins used were monomeric and the isoelectric focussing profiles confirmed the isotype specificity (Dr. T. Rademacher, personal communication). Further evidence for subclass specificity was provided by competition experiments with other monoclonal Ab of the relevant subclasses (11). Moreover, after treatment with 0.25% wt/vol trypsin at 37°C for 20 min, BCG-PM no longer bound IgG2a, but did bind both IgG2b and the Fab fragment of 2.4G2 (not shown).

Role of Lymphokines. All changes characteristic of Mϕ activation can be shown to depend upon sensitized T lymphocytes and specific Ag (8). Fig. 1 (c–f) shows that addition of lymphokine to RPM for 24 h selectively enhances expression of the IgG2a FcR. The number of EIgG2a bound/100 Mϕ increased from 140 to 620, after lymphokine, whereas EIgG2b binding decreased from 480 to 360. Scatchard analysis of monomer binding (Fig. 1, legend) confirmed that lymphokine activation increased the number of IgG2a sites by 66%, whereas IgG2b sites decreased by 13%, with little change in affinity. Addition of lymphokine to P388D1 Mϕ and J774 induced a two- to threefold increase in the number of IgG2a sites/cell (not shown).

Fc mediated O_2^- Release and Modulation. Secretion by phagocytic leukocytes of reduced products of oxygen can be triggered by phorbol myristate acetate (PMA) (7) or by contact with Ab-coated particles or aggregated IgG. Nathan et al. (3) have shown that 2.4G2 inhibits H_2O_2-dependent antibody-dependent cellular cytotoxicity (ADCC) by BCG-PM mediated by heterogeneous mouse Ab. We examined the role of different IgG subclasses in triggering release of O_2^- by plating activated Mϕ on immobilized DNP-anti-DNP complexes. Table II shows that both BCG-PM and lymphokine-activated RPM release more O_2^- on the IgG2a immune complexes, comparable to that triggered by PMA. The high spontaneous release by BCG-PM plated directly on DNP-gelatin substrates is due to adherence and spreading, and is characteristic of BCG-activated Mϕ (G. Berton, unpublished observation). Nonactivated Mϕ release little O_2^- on immune complexes, with preference for the IgG2b subclass. We have concluded that enhanced expression of IgG2a FcR by activated Mϕ correlates closely with its ability to mediate a secretory function.

Michl et al. (12) have shown that FcR can be selectively modulated from the surface of Mϕ by cultivation on immobilized immune complexes. Support for distinct FcR on activated Mϕ is provided by the evidence that the IgG2a and IgG2b receptors can be modulated independently. Fig. 1 b and Table II show that BCG-PM plated

on immune complexes of DNP-gelatin-anti-DNP (IgG2a) exhibited a 95% reduction in EIgG2a rosettes, compared with control cells on DNP-gelatin alone (Fig. 1 a). Reciprocal experiments on IgG2b immune complexes and controls are shown in Table II.

Discussion

These studies establish that at least two of the Mϕ FcR specificities can be regulated independently. Mϕ activation in vivo or in vitro selectively enhances expression of the IgG2a FcR, whereas the IgG2b receptor is reduced, as shown by binding of monomer, complexed Ab, or the monoclonal Ab, 2.4G2. Our studies favor the notion that monomer and aggregated IgG2a bind to the same FcR (1), and independent modulation of different FcR activities is compatible with the existence of two distinct Mϕ FcR molecules (15). Although BCG infection could alter the Mϕ population in the peritoneal cavity by recruitment, proliferation, and turnover, it is clear that receptor specificities can be modulated on individual Mϕ. Whereas most cells in all the populations studied express both FcR activities, the lymphokine activated RPM population provides a stable, closed system for analysis and clonal cell lines, e.g., P388D1 can be induced to show the same changes.

The effects of BCG infection can be contrasted with those evoked by an inflammatory stimulus such as thioglycollate broth, which does not induce Ia Ag or activate Mϕ cytotoxicity. Although the FcR for IgG2a was also increased in TPM, there was a concomitant increase of IgG2b expression. Reversed isotype specificity is not unique to BCG, since Trypanosoma brucei infection another Mϕ-activating stimulus (16) induces the same phenotype. T lymphocytes and lymphokines are thought to play an important role in immune mechanisms of Mϕ activation, although the nature of the inducing molecules and their mechanism of action remain obscure. Since BCG infection of nude mice induces the same FcR phenotype as in euthymic mice (unpublished), T cell-independent pathways could also activate Mϕ.

Surface expression of FcR for different isotypes correlates well with receptor activity in endocytosis and secretion of O_2^-. ADCC by BCG-PM takes place at the cell surface rather than intracellularly, and the FcR for IgG2b has been implicated in this process (3), although other workers (17) have shown that both IgG subclasses can mediate cytolysis. Release of O_2^- by BCG-PM is usually associated with generation of H_2O_2 and enhanced killing activity. Our studies indicate that the IgG2a FcR could play a major role in cytotoxicity and account for the remarkable efficacy of the homologous Ab in mediating tumor destruction in vivo (18).

Monoclonal, homologous Ab have revealed important differences in FcR structure and function which are inapparent with heterologous reagents. Since genes for the Ig isotypes (19) and their receptors are regulated with a considerable degree of independence, the role of different FcR on Mϕ and lymphoid cells in immune processes merits further consideration.

Summary

After infection with bacillus Calmette-Guérin, peritoneal macrophages (Mϕ) display enhanced expression of FcR for both monomeric and complexed IgG2a, but not IgG2b. Isotype specificity of FcR can be reversed on nonactivated Mϕ by immune lymphokines, and IgG2a immune complexes are more effective triggers of the respi-

ratory burst in activated Mϕ. Selective enhancement of Ig2a FcR by Mϕ activation could account for efficacy of homologous ab in mediating cytotoxicity in some systems.

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