

## Ontogeny of Fc Receptors and Complement Receptor (CR3) during Human Myeloid Differentiation

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**A**bstract. Two different Fc receptors for IgG (Fc<sub>γ</sub>R) have been identified on human leukocytes: a high avidity receptor (Fc<sub>γ</sub>R<sub>hi</sub>) present on monocytes but not on neutrophils, and a low avidity receptor (Fc<sub>γ</sub>R<sub>lo</sub>) present on neutrophils but not on monocytes. Fc<sub>γ</sub>R<sub>lo</sub> can be inhibited and the receptor precipitated by monoclonal antibody 3G8. We have used this monoclonal antibody to study the course of Fc<sub>γ</sub>R<sub>lo</sub> appearance on bone marrow cells, leukocytes of patients with chronic myelogenous leukemia (CML), and HL-60 and U937 cells induced to differentiate with agents such as dimethyl sulfoxide (DMSO), retinoic acid, phorbol myristate acetate, and lymphokine. We report that Fc<sub>γ</sub>R<sub>lo</sub> is a late differentiation antigen, first expressed at the metamyelocyte stage. Since precursors to metamyelocytes bear Fc<sub>γ</sub>R, and the promyelocyte line HL-60 bears Fc<sub>γ</sub>R<sub>hi</sub>, there must be a progressive loss of Fc<sub>γ</sub>R<sub>hi</sub> during myeloid differentiation and the reciprocal expression of Fc<sub>γ</sub>R<sub>lo</sub>. Results of immunoprecipitation and polyacrylamide gel analysis of the proteins are consistent with these results.

We have also studied the receptor for the C3bi complement component (CR3), which is blocked and immunoprecipitated by monoclonal antibody OKM10. During DMSO-driven differentiation of HL-60 cells, we find that CR3 is induced on all cells, whereas Fc<sub>γ</sub>R<sub>lo</sub> is

induced on only 24% of cells, suggesting that CR3 appears earlier during differentiation than Fc<sub>γ</sub>R<sub>lo</sub> does.

### Introduction

Human polymorphonuclear leukocytes (PMN) develop from bone marrow myeloid stem cells, which divide until the metamyelocyte stage, at which time they undergo terminal differentiation. PMN maturation is characterized by the acquisition of polymorphic nuclear morphology and phagocytic and microbicidal capacity. These functional changes associated with maturation are likely to be accompanied by changes in plasma membrane constituents. The quantitative expression of plasma membrane components can now be studied with the use of monoclonal antibodies as probes.

Fc receptors for IgG (Fc<sub>γ</sub>R)<sup>1</sup> play a central role in effector cell recognition of foreign antigens via cytophilic IgG. An early suggestion that there might be more than one type of Fc<sub>γ</sub>R on human leukocytes came from observations that monocytes and PMN differ in their ability to rosette erythrocytes coated with anti-Rh<sub>0</sub> sera (1). Kurlander and Batker (2) then demonstrated that PMN bind IgG1 oligomers with 100- 1,000-fold lower avidity than do monocytes. The low avidity neutrophil Fc<sub>γ</sub>R (Fc<sub>γ</sub>R<sub>lo</sub>) bears an epitope recognized by monoclonal antibody 3G8, which blocks the binding of erythrocytes sensitized with IgG (ElgG) or soluble immune complexes to PMN (3). The specificity is similar to that of monoclonal B73.1, recently isolated by Perussia et al. (4). 3G8 does not bind to monocytes, or the HL-60 or U937 cell lines, but does bind to peripheral blood neutrophils, eosinophils, most lung macrophages, and some lymphocytes. There are thus at least two receptors for IgG on human leukocytes, a low avidity receptor for immune complexes present primarily on PMN (Fc<sub>γ</sub>R<sub>lo</sub>) and a high avidity receptor on monocytes (Fc<sub>γ</sub>R<sub>hi</sub>).

1. *Abbreviations used in this paper:* ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; AMoL, acute monocytic leukemia; APL, acute promyelocytic leukemia; C, complement; CML, chronic myelogenous leukemia; Con A, concanavalin A; CR3, complement receptor for C3bi; DMSO, dimethyl sulfoxide; DNP, dinitrophenyl; ElgG, IgG coated sheep erythrocytes; Fc<sub>γ</sub>R<sub>hi</sub>, high avidity receptor for human IgG1; Fc<sub>γ</sub>R<sub>lo</sub>, low avidity immune complex receptor; FBS, fetal bovine serum; PMA, phorbol myristate acetate.

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The receptor for the C3bi complement component (CR3) has recently been identified using the monoclonal antibodies OKM1 and OKM10 (5, 6). CR3 consists of two peptides, an  $\alpha$  of 185,000  $M_r$  and a  $\beta$  of 105,000  $M_r$  and appears to be a member of a closely related family of two-chain leukocyte antigens. Each member of this family consists of a unique  $\alpha$ -chain noncovalently associated with a  $\beta$ -chain, which is identical for all members of the family (5, 6). The family includes: CR3, which is expressed on monocytes, macrophages, and PMN; LFA-1, which is associated with T cell-mediated cytotoxicity, and is found on lymphocytes, monocytes, and macrophages; and a third protein of unknown function with an  $\alpha$ -chain of 153,000  $M_r$ . All members of the family share determinants, probably on the  $\beta$ -chain, which are recognized by monoclonal antibodies IB4 and TS1/8 (5, 6).

The HL-60 cell line has been used as a model for myeloid differentiation. HL-60 cells treated with dimethyl sulfoxide (DMSO) (7) or retinoic acid (8, 9) differentiate in vitro to cells with more mature myeloid morphology and functional characteristics, whereas HL-60 cells incubated with phorbol myristate acetate (PMA) exhibit morphology more characteristic of monocytes (10). Similarly, the U937 cell line, which has monocytelike characteristics, responds to lymphokine or gamma interferon with an increased expression of  $Fc_\gamma R_{hi}$  (11–13). Myeloid precursors and mature PMN both have plasma membrane receptors for the Fc portion of IgG ( $Fc_\gamma R$ ), as demonstrated by the ability of these cells to bind EIGG (14–17). We have studied the appearance of  $Fc_\gamma R_{lo}$  and CR3 on HL-60 cells treated with inducing agents, on cells from human marrow, and on peripheral leukocytes from patients with chronic myelogenous leukemia (CML) and report that  $Fc_\gamma R_{lo}$  is a relatively late differentiation antigen.

## Methods

**Cells.** HL-60 and U937 cells were grown in alpha modified Eagle's medium (Flow Laboratories, Inc., McLean, VA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Kansas City Biological, Lenexa, KA), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (complete  $\alpha$ -minimal essential medium [ $\alpha$ -MEM]). Cells were cultured in suspension using spinner flasks, or in tissue culture plastic dishes (Nunc, Denmark).

Bone marrow samples drawn from leukemic patients for clinical evaluation were obtained from the Tumor Procurement Center of the Sloan-Kettering Institute, New York. The bone marrow suspensions (usually 2 ml) were diluted with an equal volume of phosphate-buffered saline (PBS), and then mixed with an equal volume of 6% dextran in saline (21). After standing for 30 min at 37°C, the leukocyte-rich supernatant was collected and the cells washed three times with PBS by centrifugation (200 g, 10 min).

For induction experiments, HL-60 cultures ( $1.8 \times 10^5$  cells/ml) were cultivated in complete  $\alpha$ -MEM containing either 1.25% (vol/vol) DMSO (J. T. Baker Chemical Co., Philipsburg, NJ) (7) or 1  $\mu$ M retinoic acid (Sigma Chemical Co., St. Louis, MO) (8) for a total of 6 d. HL-60 cells ( $5 \times 10^5$ /ml) were treated with 16 nM PMA (Sigma Chemical Co.) in complete medium for 2 d. Concanavalin A (Con A) supernatants were prepared by incubation of Ficoll-Hypaque-purified human mononuclear cells ( $4 \times 10^6$  cells/ml) in RPMI 1640 (Gibco Laboratories, Grand

Island, NY) containing 10% FBS and 15  $\mu$ g/ml of Con A (Miles Laboratories Inc., Elkhart, IN) (19). U937 cells ( $2 \times 10^5$ /ml) were cultivated in RPMI 1640 medium containing 25 mM Hepes, 10% FBS, and either 10% of Con A supernatant or control supernatant from untreated mononuclear cells incubated in parallel.

**Morphology and immunofluorescence.** The percentage of viable cells was determined by trypan blue exclusion. For cell differential counts,  $2-4 \times 10^5$  cells were centrifuged in a cytocentrifuge (Shandon cytopsin, Shandon Southern Instruments, Inc., Sewickley, PA) and stained with Diff-quick (Harleco, Hartman-Leddon, Philadelphia, PA). 200–300 cells were counted per sample.

For immunofluorescence staining, cells were attached to poly-L-lysine-coated glass. Either 13-mm No. 1 coverglasses (Propper Manufacturing Co., Inc., Long Island City, NY) or slides with 6-mm circles in a hydrophobic coating (Carlson Scientific, Peotone, IL) were acid washed with Chromerge (Fisher Scientific Co., Pittsburgh, PA) rinsed with water, coated with 50  $\mu$ g/ml of poly-L-lysine (70,000 mol wt, Sigma Chemical Co.) for 20 min, and rinsed in water. Cells ( $3-5 \times 10^5$ ) suspended in PBS were either centrifuged (200 g, 8 min, 4°C) onto coverglasses, or were allowed to settle (20  $\mu$ l cell suspension at  $2 \times 10^6$  cells/ml) onto the masked slides (45 min at 4°C). Unreacted cationic sites were then quenched with PBS containing 5% FBS.

Cell preparations were incubated with 3G8 Fab (50  $\mu$ g per ml) in PBS containing 5% FBS for 45 min at 4°C. The cells were rinsed in PBS-5%FBS, incubated (30 min, 4°C) with either biotinylated rabbit  $F(ab')_2$  anti-mouse IgG prepared as described (20) or a biotinylated horse anti-mouse Ig (Vector Laboratories, Burlingame, CA) at a concentration of 50  $\mu$ g/ml in PBS-5%FBS, rinsed, and stained (30 min, 4°C) with fluoresceinated Avidin-D (Vector Laboratories) at a concentration of 50  $\mu$ g/ml in PBS-5%FBS. After final rinsing, cells were fixed in buffered 10% formalin, and mounted in 90% glycerol, 10% PBS containing 1 mg/ml *p*-phenylenediamine (Aldrich Chemical Co., Inc., Milwaukee, WI), to retard fluorescence quenching (21). The *p*-phenylenediamine stains the nuclei orange, enabling accurate assessment of nuclear morphology along with the green fluorescein fluorescence. This could not be done nearly as well from the phase image of the fixed cells. Cells were examined with epifluorescence optics using a 63 $\times$  lens and photographed on Ektachrome ASA 400. Internegatives were made of the color slides on Ektapan film with a Kodak No. 25 red filter (Eastman Kodak Co., Rochester, NY).

**Fluorescence-activated cell sorter analysis.** Cells ( $2 \times 10^6$ ) were treated in suspension with identical concentrations of reagents used for immunofluorescence studies. Cells incubated with the rabbit anti- $Fc_\gamma R_{lo}$   $F(ab')_2$  were then stained with a fluoresceinated goat  $F(ab')_2$  anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). Cells were washed three times by centrifugation between each reagent. Flow microfluorometry was performed with a FACS II fluorescence-activated cell sorter (Becton, Dickinson & Co., Mountain View, CA). Exciting light at 488.8 nm was provided by a 5-W argon-ion laser (Spectra-Physics Inc., Mountain View, CA) at an incident power of 300 mW. Emitted light was optically filtered with 520-nm and 530-nm "cut-on" filters (Ditrich Optics, Marlboro, MA.) placed in series. In all cases the photomultiplier tube was set at 2/1. Fig. 2 was obtained by tracing photographs of the oscilloscopic displays, and the results presented are typical of four separate experiments. In each case controls omitting 3G8 Fab or the rabbit anti- $Fc_\gamma R_{lo}$   $F(ab')_2$ , and controls using an irrelevant Fab or rabbit  $F(ab')_2$  reagent were included. Fluorescence gain settings were 8/1 for cells stained with 3G8 or the rabbit anti- $Fc_\gamma R_{lo}$   $F(ab')_2$  and 8/0.7 for cells stained with OKM10.

**Ligands and binding studies.** Purification of monoclonal antibody and papiin Fab fragments was as described (3, 25). Human IgG1 myeloma protein (Ba) was a gift of Dr. Edward Franklin (New York Uni-

versity School of Medicine). OKM10 was the generous gift of Dr. Gideon Goldstein (Ortho Pharmaceutical, Raritan, NJ). Proteins were labeled with  $^{125}\text{I}$  using Iodo-gen (Pierce Chemical Co., Rockford, IL) as the oxidizing agent (23, 24). We routinely obtained 50–70% incorporation of label into protein, with a final specific activity of 5,000 cpm/ng protein.

A rabbit antiserum against  $\text{Fc}_\gamma\text{R}_{10}$  was prepared by injecting purified  $\text{Fc}_\gamma\text{R}_{10}$  (25) (60  $\mu\text{g}$ ) in complete Freund's adjuvant into two popliteal lymph nodes as previously described (26) and four subcutaneous sites. 19 d later, the rabbit was boosted with 20  $\mu\text{g}$   $\text{Fc}_\gamma\text{R}_{10}$ , which had been boiled in 0.1% sodium dodecyl sulfate (SDS) and emulsified in incomplete Freund's adjuvant. At the same time,  $\text{Fc}_\gamma\text{R}_{10}$  was immunoprecipitated from  $2.7 \times 10^9$  PMN with 3G8 Fab Sepharose, emulsified in incomplete Freund's adjuvant and injected subcutaneously. This procedure was performed to enhance the antibody response to sites other than that recognized by the monoclonal antibody 3G8 (27). The IgG fraction of the rabbit anti- $\text{Fc}_\gamma\text{R}$  was prepared by ammonium sulfate precipitation and purified by chromatography on a TSK-3000 column (LKB, Stockholm, Sweden). The  $\text{F(ab')}_2$  fragment was prepared by pepsin digestion and similarly chromatographed on a TSK-3000 column. The ability of  $\text{F(ab')}_2$  fragments of the anti- $\text{Fc}_\gamma\text{R}_{10}$  IgG and control rabbit IgG to inhibit binding of soluble rabbit antidiinitrophenyl (DNP) IgG-DNP<sub>3</sub>BSA complexes prepared as described (3) was tested following preincubation of the neutrophils for 1 h with the  $\text{F(ab')}_2$  preparations.

The binding of labeled 3G8 Fab or monomeric IgG1 was performed using saturating concentrations of antibody as described previously (3). Briefly, cells ( $5\text{--}20 \times 10^5$ ) were incubated (1 h,  $4^\circ$ ) in 0.1 ml of PBS containing 1 mg/ml bovine serum albumin (PBS-BSA) and 2  $\mu\text{g}$ /ml of labeled ligand (2,000–4,000 cpm/ng protein). To remove unbound ligand the cells were diluted with 2.5 ml PBS-5%FBS, and the suspension was centrifuged. The cell pellet was resuspended in 1 ml of PBS-5%FBS, centrifuged, and the radioactivity in the cell pellet was determined. Results were corrected for nonspecific binding by subtraction of radioactivity bound using an equivalent input of radioactivity in labeled ligand of low specific activity (10 cpm/ng).

**Cell labeling and immunoprecipitation.** Cells were labeled with  $^{125}\text{I}$  using lactoperoxidase and glucose oxidase (28). The labeled cells were lysed in PBS containing 0.5% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.28 IU aprotinin/ml (29). 3G8 Fab, IB4 IgG, and monomeric IgG1 were coupled to CNBr-activated Sepharose 4B at 2–4 mg protein/ml of Sepharose. The rabbit anti- $\text{Fc}_\gamma\text{R}_{10}$

serum prepared by immunization with purified  $\text{Fc}_\gamma\text{R}$  (25) was adsorbed to fixed *Staphylococcus aureus* (Zymed Laboratories), which were then washed by centrifugation in the lysis buffer. The lysate of the labeled cells, after clearing (40,000 g, 30 min), was incubated (1 h,  $4^\circ\text{C}$ ) on a shaker with the immunoadsorbents, which were then washed twice with a high salt buffer (0.6 M NaCl, 0.0125 M  $\text{KPO}_4$ , pH 7.4, 0.02%  $\text{NaN}_3$ ), twice with a mixed micelle buffer (0.05% NP-40, 0.1% SDS, 10 mM Tris, pH 8.6, 0.3 M NaCl), and again with the high salt buffer (29, 30). Adsorbed protein was eluted with sample buffer and electrophoresed on 1-mm-thick slab gels with a 4–11% polyacrylamide gradient (31). The dried gels were exposed at  $-70^\circ\text{C}$  on Kodak X-Omat AR film using enhancing screens (DuPont Instruments, Wilmington, DE). The results presented are typical of at least four experiments.

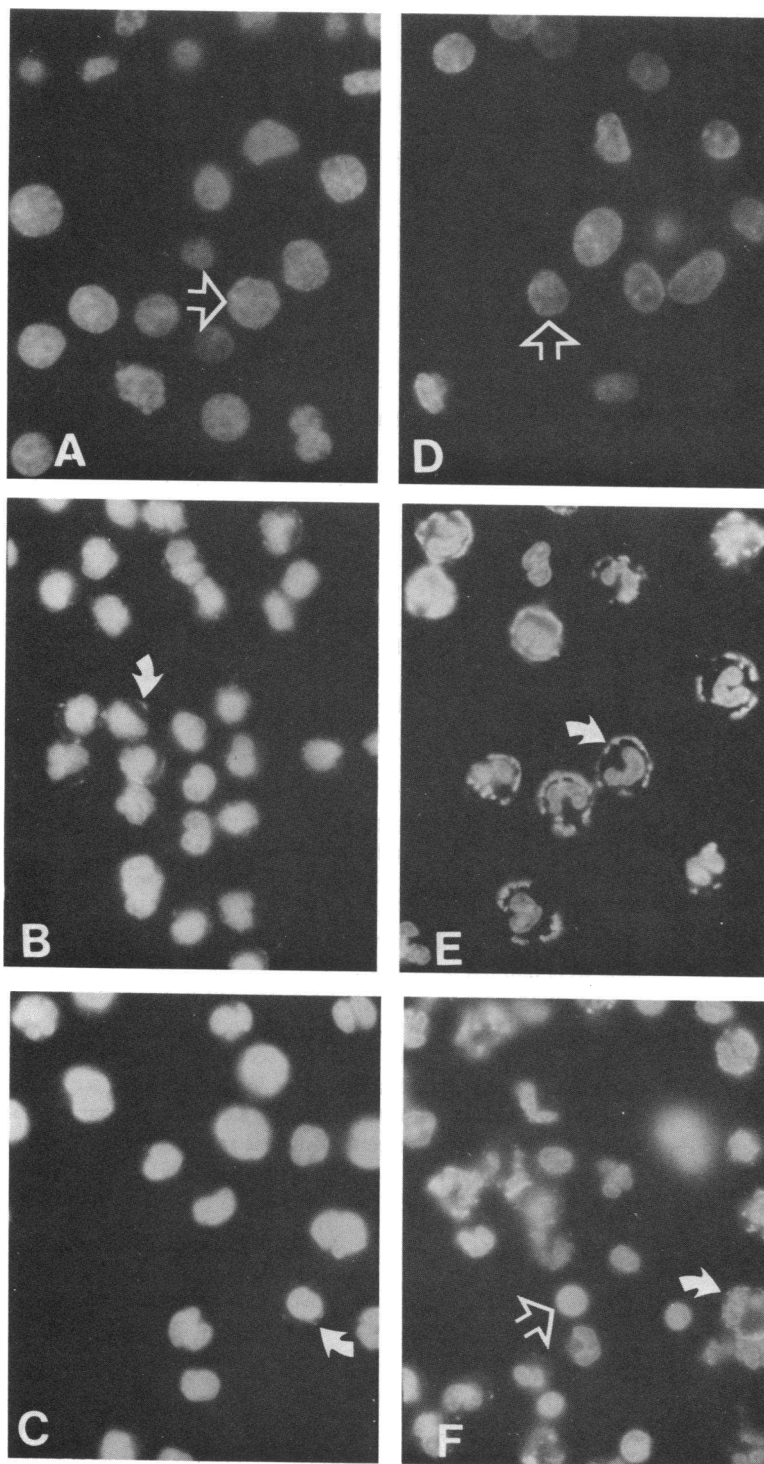
## Results

**Induction of  $\text{Fc}_\gamma\text{R}_{10}$  on HL-60 cells.** The HL-60 cell line has been widely used as a model for myeloid differentiation since culture with compounds such as retinoic acid and DMSO results in the differentiation of these cells to more “mature” forms (7–9). In agreement with previously published reports (7), we found >95% of uninduced HL60 cells resembled promyelocytes. Culture with DMSO and to a lesser extent with retinoic acid, resulted in the appearance of a large percentage of cells resembling metamyelocytes, and a small percentage of more mature forms (Table I). We examined by immunofluorescent staining HL-60 cells that had been induced to differentiate and found a correlation between the extent of differentiation as judged by morphology and expression of  $\text{Fc}_\gamma\text{R}_{10}$  (Table I, Fig. 1). Incubation with DMSO (Fig. 1 B) and retinoic acid (Fig. 1 C) resulted in fluorescent staining by 3G8 on 24.3% and 5.2% of the cells, respectively, compared with <0.2% of the uninduced population (Table I, Fig. 1). Incubation of the HL-60 cells with PMA did not result in staining with 3G8 (Table I, Fig. 1 D). The few positive cells present in the uninduced population of HL-60 cells stained with 3G8 had metamyelocyte nuclear morphology and had matured spontaneously without the addition of any drug (7) (Table I).

Table I. Effect of Inducers on  $\text{Rf}_\gamma\text{R}_{10}$  and IB4 Antigen Expression by HL-60 and U937 Cells

Cell type	Treatment	Cell differential			Band	PMN	3G8 staining cells	IB4 staining cells
		Promyelocyte	Myelocyte	Metamyelocyte				
		%	%	%	%	%	%	%
HL-60	None	99.7	0.2	0.1	0	0	0.17±0.11 (n = 23)	99
	DMSO	0.9	76.2	21.3	1	0.6	24.3±10.1 (n = 23)	99
	Retinoic acid	1.3	89.4	8.9	0.5	0	5.2±3.5 (n = 10)	99
	PMA			ND			0.25±0.14 (n = 8)	99
U937	None			ND			0 (n = 4)	31
	10% Con A supernatant			ND			0 (n = 4)	99

Differentials were determined by Diff-Quik staining as described. 3G8- and IB4-positive populations were determined by immunofluorescent staining. Concentrations of inducers were DMSO, 1.25% (vol/vol); retinoic acid, 1  $\mu\text{M}$ ; PMA, 16 nM.



**Figure 1.** Immunofluorescent staining by 3G8 of HL-60 cells, peripheral blood PMN, and bone marrow leukocytes. Cells were prepared as described in the Methods section. (A) HL-60 cells. (B) HL-60 cells treated with DMSO. (C) HL-60 cells treated with retinoic acid. (D) HL-60 cells treated with PMA. (E) PMN. (F) Bone marrow leukocytes. The solid arrows point out membrane fluorescence (green in the original transparency) and the hollow arrows indicate nuclear fluorescence (orange in the original).

In agreement with the results obtained by immunofluorescence, quantitative binding studies using  $^{125}\text{I}$ -labeled 3G8 Fab and monomeric IgG1 showed there was no detectable binding

of 3G8 Fab on uninduced HL-60 cells (3) nor on PMA-treated HL-60 cells (Table II). In contrast, significant binding of  $^{125}\text{I}$ -3G8 Fab was demonstrated on both DMSO- and retinoic acid-

Table II. Quantitative Binding of 3G8 Fab and Human IgG1 to Control and Induced HL-60 and U937 Cells

Cell type	Treatment	3G8 Fab*		IgG1‡	
		Number of sites per 3G8-positive cell		Average number of sites	
HL-60	None	0	(n = 12)	3.37±1.86 × 10 <sup>3</sup>	(n = 12)
	DMSO	3.14±1.13 × 10 <sup>4</sup>	(n = 10)	4.00±1.43 × 10 <sup>3</sup>	(n = 11)
	Retinoic acid	3.64±0.82 × 10 <sup>4</sup>	(n = 7)	8.45±2.6 × 10 <sup>3</sup>	(n = 8)
	PMA	0	(n = 5)	1.25±2.0 × 10 <sup>4</sup>	(n = 5)
U937	None	0	(n = 3)	8.3±2.5 × 10 <sup>3</sup>	(n = 5)
	10% Con A supernatant	0	(n = 3)	2.2±0.7 × 10 <sup>4</sup>	(n = 5)

Concentrations of inducers were as in Table I. \* Binding values are corrected to determine the number of sites per positive cell. ‡ Binding values represent population averages.

treated cells. Furthermore, when binding data were analyzed with respect to the percentage of positive cells (Table I) a comparable number of 3G8 Fab-binding sites per positive cell was found for both the DMSO- and retinoic acid-induced populations (Table II).

HL-60 cells, unlike peripheral neutrophils, have a high avidity Fc<sub>γ</sub>R for monomeric human IgG1 (Fc<sub>γ</sub>R<sub>hi</sub>) (32). Binding of IgG1 to DMSO-induced HL-60 cells was comparable to un-induced cultures (Table II). Incubation of HL-60 cells with PMA, however, resulted in a threefold increase in the amount of IgG1 bound.

**Effect of lymphokine on Fc<sub>γ</sub>R expression by U937 cells.** The U937 cell line is a monocytelike line that responds to treatment with lymphokine preparations or gamma interferon with an increase in Fc<sub>γ</sub>R<sub>hi</sub> expression (12, 13). We examined whether the expression of Fc<sub>γ</sub>R<sub>lo</sub> could be induced on U937 cells following culture for 48 h in medium containing 10% of a supernatant from mononuclear cells treated with Con A. Immunofluorescence studies of untreated or lymphokine-treated U937 cells failed to demonstrate any cells expressing Fc<sub>γ</sub>R<sub>lo</sub> (Table I). In contrast, the binding of <sup>125</sup>I-IgG1 to the lymphokine-treated U937 cells increased by almost threefold (Table II), in agreement with the results of Guyre et al. (12, 13).

**Expression of CR3 on HL-60 and U937 cells.** The monoclonal antibody OKM10 recognizes CR3 of human leukocytes (5). We examined the expression of this receptor on HL-60 cells and on HL-60 cells induced to differentiate with DMSO or retinoic acid. HL-60 cells express no CR3 as assessed by the binding of OKM10 (Fig. 2 C). However, treatment with DMSO or retinoic acid resulted in the expression of this receptor on all cells as determined by fluorescence-activated cell sorter analysis (Fig. 2 C).

We also examined the expression of antigens recognized by the antibody IB4, which recognizes a determinant shared by at least three members of a family of two-chain leukocyte membrane proteins (5, 6). All HL-60 cells and 30% of U937 cells expressed low levels of fluorescent staining with IB4 (Table I). However, after incubation with lymphokine, >99% of U937 cells were stained with IB4.

To quantitate our results on a per cell basis, we also performed cell sorter analysis on drug-induced and control HL-60 populations (Fig. 2 A and B). Fluorescence-activated cell sorter analysis demonstrated a shift to the left in the light scatter of the DMSO-treated HL-60 cells indicative of the appearance of smaller cells. The fluorescence analysis revealed that 22.3% of the DMSO-treated cells were 3G8 positive (Fig. 2 A).

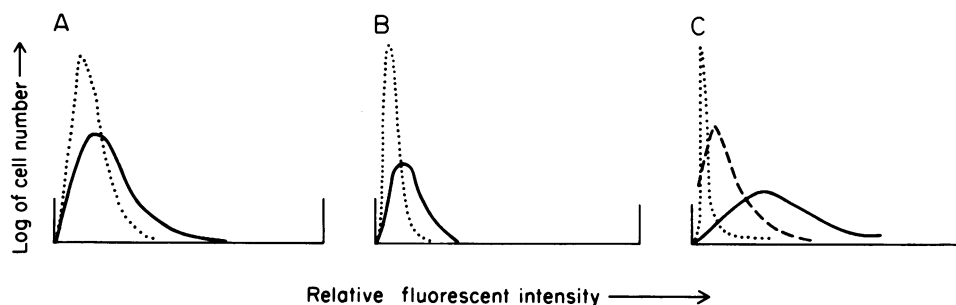


Figure 2. Fluorescence-activated cell sorter analysis of HL-60 or DMSO-induced HL-60 cells stained with 3G8 Fab, rabbit anti-Fc<sub>γ</sub>R<sub>lo</sub>, and OKM10. (A) Binding of 3G8 to HL-60 cells (· · · · ·) and HL-60 cells treated with DMSO (—). (B) Binding of rabbit anti-Fc<sub>γ</sub>R<sub>lo</sub> F(ab')<sub>2</sub> to HL-60 cells (· · · · ·) and HL-60 cells treated with DMSO (—). (C) Binding of OKM10 to HL-60 cells (· · · · ·), HL-60 cells treated with retinoic acid (-----), or HL-60 cells treated with DMSO (—).

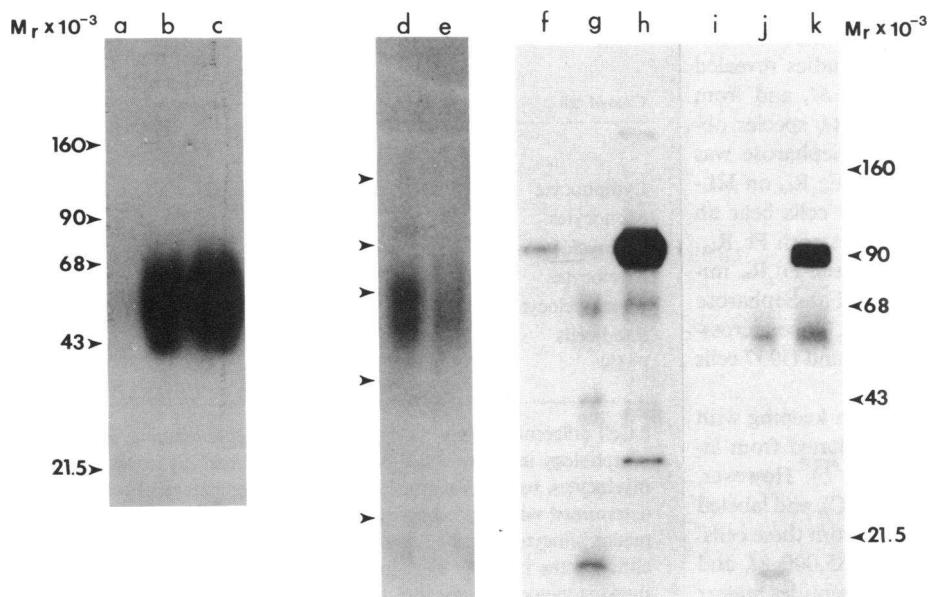
Cell sorter analysis was also performed with rabbit anti-Fc $\gamma$ R $\alpha$  F(ab') $_2$ , obtained by immunizing a rabbit with purified Fc $\gamma$ R $\alpha$  obtained from neutrophils by affinity chromatography. This antiserum immunoprecipitated the same protein from labeled cells as the monoclonal antibody (Fig. 3, lanes b-e). Furthermore, F(ab') $_2$  fragments of the antiserum inhibited the binding of soluble immune complexes to peripheral neutrophils, whereas control rabbit F(ab') $_2$  had no effect (Fig. 4). When the rabbit anti Fc $\gamma$ R $\alpha$  serum was used to stain DMSO-treated HL-60 cells, a slightly higher percentage of staining was found (31.9%) than with 3G8 Fab, suggesting that this antisera might cross-react with epitopes on other Fc receptors (Fig. 2 B). We also found that only a fraction of DMSO-induced HL-60 cells stained with 3G8 whereas the same cells stained uniformly with OKM10 (Fig. 2 C).

**Expression of the 3G8 and IB4 antigens by cells from CML patients or leukemic bone marrow.** Another model for neutrophil maturation is found in patients with CML. In the peripheral circulation of these patients cells with mature neutrophil morphology as well as less differentiated cells are found. We reported previously that the less dense (immature) cells had fewer binding sites for 3G8 (3). Immunofluorescence studies (Table III) confirm this and demonstrate that cells exhibiting nuclear morphology earlier than the metamyelocyte stage lack Fc $\gamma$ R $\alpha$ , while the cells at or beyond the metamyelocyte stage stain with essentially the same fluorescence intensity as peripheral neutrophils. In parallel with immunofluorescence studies for Fc $\gamma$ R $\alpha$ , we also examined IB4 antigen expression on leukocytes from CML patients. Of the peripheral leukocytes from two patients, 14 and 68% were positive for 3G8; 68 and 76% respectively, were positive for IB4. Some lymphocytes and blastlike cells were IB4-negative,

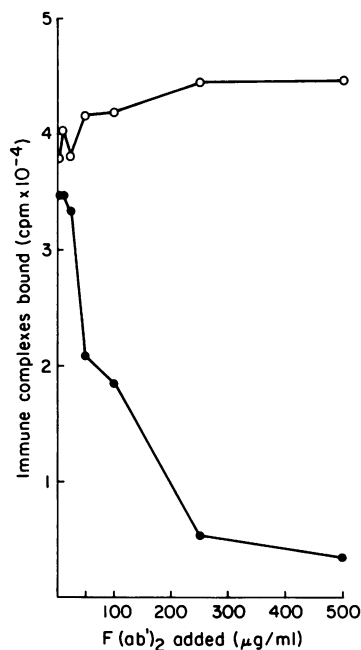
but it is clear that IB4 stains a larger percentage of immature myeloid precursors than 3G8 does.

A series of bone marrow samples from patients with leukemia or in remission (acute myelogenous leukemia [AML], acute promyelocytic leukemia [APL], acute monocytic leukemia [AMoL], and acute lymphocytic leukemia [ALL]) was examined in order to determine the stage of myeloid development at which the antigen recognized by 3G8 could be detected. During preliminary experiments, we made the fortuitous observation that inclusion of *p*-phenylenediamine in the mounting fluid, which dramatically slows the rate of fluorescence bleaching (21), resulted in orange staining of nuclei. This enabled us to score accurately 3G8 Fab staining and nuclear morphology on the same cells. We found that only cells at the metamyelocyte stage of development or beyond expressed Fc $\gamma$ R $\alpha$  (Table IV). Indeed, the intensity of fluorescence was greatest on cells with fully mature nuclear morphology (Fig. 1 F). Cells with round or blastlike nuclear morphology did not demonstrate any fluorescent staining.

**Immunoprecipitation of Fc $\gamma$ R.** To extend the above results, we performed immunoprecipitation studies on  $^{125}$ I-labeled cells with monoclonal and polyclonal sera and analyzed the labeled proteins by SDS-polyacrylamide gel electrophoresis. Immunoprecipitation of NP-40 lysates from surface-labeled DMSO-treated HL-60 cells and peripheral blood PMN with either 3G8 Fab or a rabbit anti-Fc $\gamma$ R $\alpha$  antiserum yielded identical proteins with broad electrophoretic mobility, from 50,000 to 74,000  $M_r$  and from 56,000 to 74,000  $M_r$ , respectively (Fig. 3, lanes b-e), confirming that the two sera react with the same protein. The rabbit antisera to Fc $\gamma$ R $\alpha$  precipitated a larger amount of this polypeptide than did 3G8 Fab. As expected on the basis of



**Figure 3.** Polyacrylamide gel electrophoresis of iodinated proteins isolated from peripheral blood PMN, HL-60 cells, HL-60 cells treated with DMSO, and U937 cells. Lysates of PMN are in lanes a-c; lysates of HL-60 cells treated with DMSO are in lanes d and e; lysates of HL-60 cells are in lanes f-h; and lysates of U937 cells are in lanes i-k. Lanes a, h, and k: lysates subjected to affinity chromatography on IgG1-Sepharose; lanes b, d, g, and j: lysates immunoprecipitated with a rabbit anti-Fc $\gamma$ R $\alpha$  serum adsorbed to *S. aureus*; lanes c, e, f, and i: lysates immunoprecipitated with 3G8 Fab-Sepharose. Molecular weight markers are: 160,000, *Escherichia coli* RNA polymerase alpha subunit; 90,000, *E. coli* RNA polymerase beta subunit; 68,000, BSA; 43,000, ovalbumin; 21,500, soybean trypsin inhibitor.



**Figure 4.** Inhibition of binding to neutrophils of soluble immune complexes by  $F(ab')_2$  anti- $Fc\gamma R_{10}$ . Soluble immune complexes of rabbit anti-DNP IgG-DNP<sub>6</sub>BSA at a final concentration of 5  $\mu$ g IgG/ml were added to neutrophils ( $10^6$ /sample) that had been preincubated for 1 h with  $F(ab')_2$  as noted. After 1 h of incubation the cells were washed by centrifugation. (○) control  $F(ab')_2$ ; (●) rabbit anti- $Fc\gamma R_{10}$   $F(ab')_2$ . All incubations were carried out at 4°C.

immunofluorescence data (Figs. 1 and 2), 3G8 failed to precipitate 50,000- to 74,000- $M_r$  proteins from untreated HL-60 or U937 cells (Fig. 3, lanes *f* and *i*).

Affinity chromatography of NP-40 lysates from HL-60 or U937 cells on IgG1-Sepharose revealed polypeptides migrating from 62,000 to 68,000  $M_r$  and from 48,000 to 63,000  $M_r$ , respectively, and a peptide centered at 90,000  $M_r$  (Fig. 3, lanes *h* and *k*). No labeled protein was isolated from PMN lysates in parallel experiments (Fig. 3, lane *a*). We carried out immunoprecipitation studies on HL-60 and U937 cell lysates using the rabbit anti- $Fc\gamma R_{10}$  serum to determine if the serum cross-reacted with the  $Fc\gamma R_{hi}$  on these cells. These studies revealed broad bands migrating from 64,000 to 71,000  $M_r$  and from 48,000 to 55,000  $M_r$ , respectively; the 90,000- $M_r$  species observed after affinity chromatography on IgG1-Sepharose was absent (Fig. 3, lanes *g* and *j*). Since there is no  $Fc\gamma R_{10}$  on HL-60 or U937 cells, these data suggest that these cells bear an  $Fc\gamma R$  that shares some but not all antigenic sites with  $Fc\gamma R_{10}$ . The similarity on SDS-polyacrylamide gels between  $Fc\gamma R_{10}$  immunoprecipitated from neutrophils with 3G8 Fab-Sepharose and the polypeptide isolated using rabbit anti- $Fc\gamma R_{10}$  sera cross-reacting with determinants on uninduced HL-60 and U937 cells is noteworthy.

Uninduced HL-60 cells bear no CR3, and in keeping with this observation, no protein was immunoprecipitated from labeled HL-60 cells using OKM10 (Fig. 5, lane *b*). However, DMSO-induced HL-60 cells express CR3 (Fig. 2 C), and labeled polypeptides were readily immunoprecipitated from these cells. Two peptides were observed, an  $\alpha$ -peptide of 185,000  $M_r$  and a  $\beta$ -peptide of 105,000  $M_r$  (Fig. 5, lane *c*). These peptides appear

identical to CR3 of human macrophages. IB4 recognizes a family of proteins, of which CR3 is a member. Uninduced HL-60 cells express only one member of that family, a species with an  $\alpha$ -chain of 190,000  $M_r$  and a  $\beta$ -chain of 105,000  $M_r$  (Fig. 5, lane *e*). This species, therefore, is probably LFA-1. In contrast, induced HL-60 cells express three members of the family recognized by IB4. IB4 precipitates the 190,000  $M_r$   $\alpha$ -chain expressed in uninduced cells, the 185,000  $M_r$   $\alpha$ -chain of CR3, and a 153,000- $M_r$  chain, as well as the 105,000- $M_r$   $\beta$ -chain. These data suggest that the maturation of HL-60 cells is accompanied by the *de novo* expression of two members of the family recognized by IB4, CR3, and the uncharacterized species with a 153,000- $M_r$   $\alpha$ -chain.

## Discussion

Our results demonstrate that  $Fc\gamma R_{10}$ , recognized by monoclonal 3G8, appears late in myeloid development. Immunofluorescence studies of human leukemic bone marrow and peripheral blood leukocytes from patients with CML provide evidence that the antigen first appears at the metamyelocyte stage. These findings were extended by studies of the promyelocyte cell line HL-60, which does not bind 3G8 Fab. Induction of this cell line toward myeloid differentiation by DMSO or retinoic acid resulted in the expression of  $Fc\gamma R_{10}$  on a variable percentage of the population. Uninduced HL-60 cells express a high avidity Fc receptor for IgG1. Unfortunately, the IgG1 binding data we have amassed from control and DMSO-induced HL-60 cells are population averages, so we do not know whether the induced cells which

**Table III.** Expression of  $Fc\gamma R_{10}$  on Leukocytes from CML Patients

Class of cell	Patient R.G.		Patient S.H.	
	*Differential counts	3G8 positive	Differential counts	3G8 positive
	% of total	% of total	% of total	% of total
Lymphocyte	5	0	32	1
Monocytes	2		2	
Promyelocytes	18		19	
Myelocytes	4		38	
Metamyelocytes	19	16	4	7
Band cells	17	12	2	2
PMN	35	40	3	5

\* Cell differentials were determined by Diff-Quik staining. By nuclear morphology in fluorescence microscopy we could distinguish metamyelocytes, band cells, and PMN, and the percentage of cells thus determined was in good agreement with the Diff-Quik results. All the metamyelocytes, band cells, and PMN expressed  $Fc\gamma R_{10}$ , although the intensity of staining differed, with the more mature cells showing greater fluorescence intensity.



Table IV. Expression of  $Fc\gamma R_{10}$  on Leukocytes from Bone Marrow\*

Class of cell‡	AML	APL§	APL§	AMoL§	AMoL	ALL§	ALL§	Mean values
								%
Nonsegmented nucleus	0	0	0	0	0	0	0	0
Metamyelocyte	5.4	8.6	7.2	4.7	4.8	4.3	3.7	5.5
Band cell	10.8	6.6	6.6	11.1	6.8	1.4	6.0	7.0
Polymorphonuclear	48.5	53.0	45.2	26.9	44.9	17.9	24.6	37.3

\* Data are expressed as percent positive cells staining with 3G8 Fab. ‡ Nuclear morphology was determined by immunofluorescence as described. § During remission.

express  $Fc\gamma R_{10}$  also bind IgG1 with high avidity. However, since it is clear that myeloid cells before the metamyelocyte stage express Fc receptors (14, 33–35) but do not bind 3G8 IgG or Fab, and mature neutrophils lack the high avidity  $Fc\gamma R_{hi}$ , there must be loss of  $Fc\gamma R_{hi}$  during maturation. Whether this occurs by shedding from the plasma membrane, turnover and lack of replacement, or represents active loss of receptor due to degradation remains to be determined.

We have also used the inducible HL-60 cell line to order the sequence of appearance of new antigenic determinants. The expression of  $Fc\gamma R_{10}$ , as we have discussed, is a relatively late differentiation antigen. CR3, as measured by binding of OKM10, appears at an earlier stage of development (36), since after induction with DMSO >99% of HL-60 cells are OKM10 positive as compared with 24.3% that express  $Fc\gamma R_{10}$ .

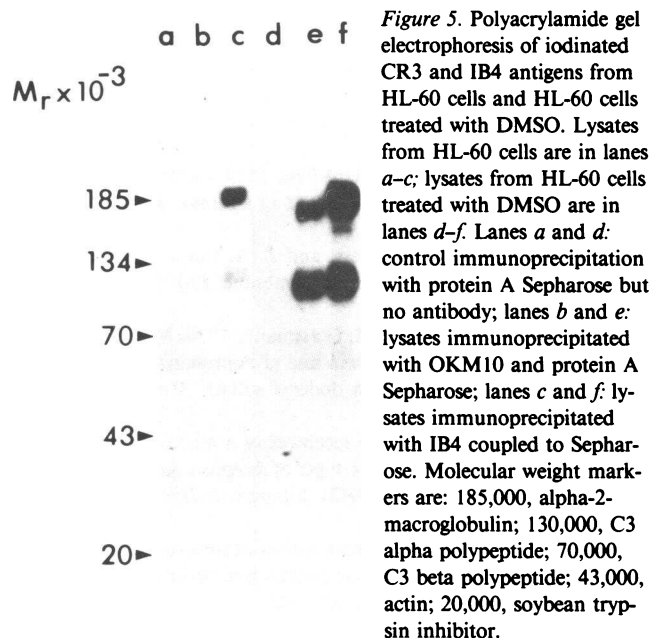
We have also studied the induction of Fc receptors on the U937 line following lymphokine treatment and on HL-60 cells

following incubation with PMA. PMA treatment of HL-60 results in a threefold increase in IgG1 binding but no induction of  $Fc\gamma R_{10}$ . Guyre et al. (12) reported that the IgG1 binding capacity of U937 cells was stimulated six- to eightfold by mixed leukocyte culture supernatants and by cloned gamma interferon (13). We demonstrate here that IgG1 binding by U937 is stimulated threefold by Con A supernatant, but find that there is no induction of  $Fc\gamma R_{10}$ , demonstrating the independent regulation of the two Fc receptors. Similar independent regulation of mouse Fc receptors specific for IgG2b/IgG1 immune complexes and IgG2a has been reported (37–39).

There are several reports of purification of human Fc receptors in the general range of 40,000–70,000  $M_r$  (40–42) by affinity chromatography on columns of immobilized IgG. We have explored the heterogeneity of human Fc receptors using affinity chromatography on IgG1-Sepharose, immunoprecipitation with monoclonal anti- $Fc\gamma R_{10}$  antibody 3G8, and immunoprecipitation with a rabbit serum elicited by 3G8-affinity purified  $Fc\gamma R_{10}$ . As one might predict from the low avidity of the PMN  $Fc\gamma R_{10}$  for IgG1 (2, 3), we find nothing precipitated by affinity chromatography of PMN lysates on IgG1-Sepharose. However, from uninduced HL-60 and U937 cells, in agreement with others (40–42), we find a protein with broad electrophoretic mobility of ~60,000  $M_r$ , as well as a major peptide with a  $M_r$  of 90,000. The rabbit anti- $Fc\gamma R_{10}$  serum appears to cross-react with the  $Fc\gamma R_{hi}$  on uninduced HL-60 and U937 cells since it immunoprecipitates the same diffuse protein of ~60,000  $M_r$  observed following affinity chromatography on IgG1-Sepharose. The 90,000  $M_r$  peptide observed following affinity chromatography on IgG1-Sepharose is not immunoprecipitated by the rabbit anti- $Fc\gamma R_{10}$  serum. The identity of this protein is unclear. These results demonstrate the independence of  $Fc\gamma R_{10}$  and  $Fc\gamma R_{hi}$  but also underscore the probability that these are closely related proteins, which could have been inferred previously only by the striking similarity of the isolated proteins upon polyacrylamide gel electrophoresis.

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