Novel Fcγ Receptor I Family Gene Products in Human Mononuclear Cells

Andrew J. Porges, Patricia B. Redecha, Robert Doebele, Lydia C. Pan, Jane E. Salmon, and Robert P. Kimberly
The Hospital for Special Surgery, Cornell University Medical College, New York 10021

Abstract

Unlike the human FcγRII and FcγRIII families, which exhibit considerable diversity at both the nucleic acid and protein levels, the human FcγRI family has only a single recognized product expressed as a 70-kD cell surface receptor with high affinity for monomeric IgG (hFcγRIa1). Using both polymerase chain reaction–based amplification and Northern hybridization, we document multiple interferon-γ–inducible hFcγRI RNA transcripts in human mononuclear cells and neutrophils. The sequences of two of these FcγRI related transcripts indicate that they are alternatively spliced products of a second FcγRI family gene, termed FcγRIB. The cDNA derived from the larger of these transcripts, termed hFcγRIb1, encodes a surface molecule that is not recognized by FcγRI specific monoclonal antibodies when transfected into COS-7 cells. Unlike the interferon-γ–inducible hFcγRIA gene product, hFcγRIb1 does not bind monomeric IgG with high affinity. However, both hFcγRIa1 and hFcγRIb1 do bind aggregated human IgG. Previously unrecognized diversity within the hFcγRI family includes an interferon-γ–inducible, putative low affinity Fcγ receptor that may play an important role in the mechanism by which Fcγ receptors participate in the humoral immune response. (*J. Clin. Invest.* 1992. 90:2102–2109.) Key words: immunoglobulin • interferon-γ • mRNA/cDNA • high affinity

Introduction

Among the three families of human Fcγ receptors (hFcγRs),[1] the hFcγRI family is the most distinct (1–8). Although hFcγRI appears to have diverged from a primordial FcγR gene early in evolution before the gene duplication and recombination leading to the hFcγRII and hFcγRIII families (8), FcγRI lacks the recognized structural diversity of these two families. The hFcγRII family consists of at least three distinct genes, two of which have multiple splice isoforms (6). The hFcγRIII family has two distinct genes encoding highly homologous proteins which demonstrate cell type–specific expression and different membrane anchors (7). In contrast, hFcγRI has only one recognized family member (9–11).

While the hFcγRII and hFcγRIII families encode receptors with low affinity for IgG (1–8), the hFcγRI family contains the only FcγR with high affinity for monomeric IgG (9–11). This increased affinity presumably results from the presence of a third extracellular immunoglobulin-like domain (EC3) not present in other FcγRs (12). Similarly, the cytoplasmic domain of hFcγRI shows limited homology with other FcγRs and appears to have a distinctive capacity to interact intracellularly with actin binding protein (13). Finally, the expression of hFcγRI is regulated by IFN-γ (14–16) through a cis acting IFN-γ response region within the promoter (17). Each of these elements suggests an important role for the hFcγRI family in the humoral immune system.

Preliminary evidence has suggested the possibility that our current understanding of the hFcγRI family may be incomplete, and that one or two additional hFcγRI genes may exist (hFcγRIB, hFcγRIC) (18, 19). Three highly homologous cDNA clones, each encoding 70-kD surface glycoproteins with three extracellular immunoglobulin-like domains and with high affinity for monomeric IgG have also been described (20, 21). However, there has been no evidence that any FcγRI gene product, other than the full-length transcript predicted by the hFcγRIA gene, encodes a functional IgG receptor on the surface of cells.

We report the existence of three distinct transcripts from the hFcγRI family in human monocytes. One transcript is a full-length product of the hFcγRIA gene, encoding a surface molecule with high affinity for monomeric IgG and recognized by mAbs specific for the 70-kD FcγRI. Two other transcripts encode a number of nonconservative amino acid substitutions, matching preliminary reports of the hFcγRIB genomic sequence (19). One of these hFcγRIB gene transcripts lacks the entire exon encoding the third extracellular immunoglobulin-like domain (EC3), while the other transcript lacks sequences encoding both EC1 and EC3. When transfected into COS-7 cells, the longer of these two putative hFcγRIB gene transcripts does not bind monomeric IgG, but does bind aggregated human IgG. This lower affinity FcγR, encoded by the IFN-γ inducible hFcγRIB gene and sensitive to multivalent immunologic stimuli such as immune complexes, may play a significant role in triggering leukocytes and modulating immune effector functions.

Methods

Cell isolation and culture. Briefly, 15 ml of heparinized fresh human whole blood was diluted with 15 ml of HBSS (Gibco Laboratories,

Address correspondence and reprint requests to Andrew J. Porges, M.D., The Hospital for Special Surgery, 535 East 70th St., New York, NY 10021.

Received for publication 23 June 1992 and in revised form 7 August 1992.

1. *Abbreviations used in this paper:* EA, erythrocytes coated with rabbit IgG; E_{BA}, streptavidin-coated erythrocytes; E-hIgG, erythrocytes coupled to human IgG; hFcγR, human Fcγ receptor; hIFN-γ, human interferon-γ; PCR, polymerase chain reaction; RT, reverse transcriptase.

J. Clin. Invest.

0021-9738/92/11/2102/08 $2.00
Volume 90, November 1992, 2102–2109

Grand Island, NY) and separated by density gradient centrifugation in sterile Ficoll-Hypaque (22). Mixed mononuclear cells were washed three times, resuspended in RPMI 1640 containing 20% heat inactivated autologous serum, plated on 100-mm plastic dishes (Becton Dickinson, Morristown, NJ) and incubated at 37°C for 6 h. Nonadherent cells were removed by gentle washing. Those cells receiving recombinant hIFN-γ (Genzyme Corp., Cambridge, MA) were treated with 200 U/ml for 6 h before harvest (16).

The human monocytoid cell line U937 (ATCC CRL 1593) was cultured in RPMI 1640 supplemented with IgG depleted 10% FCS (Hyclone Laboratories, Inc., Logan, UT), 2 mM glutamine, 25 mM Hepes, and penicillin-streptomycin in a humidified atmosphere containing 5% CO_2. COS-7 cells (ATCC CRL 1651) were cultured under the same conditions in DMEM with the same supplements.

RNA extraction and polymerase chain reaction. 5×10^7 purified mononuclear cells or U937 cells were lysed with 4 M guanidinium thiocyanate (Boehringer Mannheim Biochemicals, Indianapolis, IN) buffer containing 1% β-mercaptoethanol and RNA was isolated by ultracentrifugation at 35,000 rpm for 18 h over a 5.7 M cesium chloride gradient (23). Total cellular RNA was then recovered by ethanol precipitation.

For cDNA synthesis, 10 μg RNA was incubated with 50 pM an "antisense" oligonucleotide primer complementary to the 3′ noncoding region of p135 (GAA GTC AGT TCA TTT ATT TGC) (20), 100 U MuMLV reverse transcriptase (RT) (U.S. Biochemical Corp., Cleveland, OH) and 200 μM each dNTP for 60 min.

The polymerase chain reaction (PCR) was performed by adding to cDNA 50 pM "sense" primer from the 5′ noncoding region of p135 (GAC AGA TTT CAC TGC TCC CAC CAG), 2 U *Taq* polymerase, and increasing reaction volume to 100 μl with PCR buffer (Perkin-Elmer Cetus, Norwalk, CT) and 1.5 mM $MgCl_2$ (24). In a thermal cycler, the mix then underwent 30 cycles of denaturation at 94°C for 1 min annealing at 50°C for 1 min and extension at 72°C for 3 min. No visible product was seen with ethidium bromide staining of products at the end of this first series of PCR amplifications.

To increase amplification, a nested PCR approach was used (Fig. 1 *A*). A second set of sense (AGA CAA CAT GTG GTT CTT GA) and antisense (CT TTA AGA GTT ACA TAC CAT) primers ~ 30 bp within the initial set of primers were synthesized. The product of the first 30 cycles of PCR was diluted 1:50 in 1 × PCR buffer, plus 200 μM free nucleotides, 50 pmol of each of the inner primers, 1.5 mM $MgCl_2$, and 2 U *Taq* polymerase. Thermal cycling was then performed for 20 cycles under similar conditions, except that annealing was performed at 54°C to increase specificity.

For direct DNA sequencing of PCR products, several other primers were selected to amplify smaller cDNA fragments. These primers were homologous to each of the three extracellular domains of p135 (EC1 sense: ACA GCC ACT CAG ACC TCG AC, EC1 antisense: TGT GGA TTT CCA GCT GTA TG, EC2 sense: GTA CAA TGT GCT TTA CTA TCG, EC2 antisense: GAG GTT AGA ATT CCA GTG GA, EC3 sense: GCT CCA GTG CTG AAT GCA TC, EC3 antisense: ACT CAG GGC TGC GCT TAA GG). When used in combination, these primers generated overlapping 300–700 bp cDNA products.

Cloning and sequencing. PCR-amplified products from freshly isolated human mononuclear cells were separated by electrophoresis through 2% low melting point agarose, and each band was excised and purified using a silica matrix based Geneclean kit (BIO 101, Inc., La Jolla, CA). Each PCR product was phosphorylated with T4 polynucleotide kinase (U.S. Biochemical Corp.) and ATP. Vector pRc/CMV (Invitrogen, San Diego, CA) was linearized, converted to blunt ends with T4 polymerase, dephosphorylated with calf intestinal phosphatase, and ligated to PCR products using T4 DNA ligase (25). Ligated vectors were used to transform competent *Escherichia coli.*

For sequencing, cloned PCR products were denatured in 0.2 M NaOH for 30 min, annealed to 0.5 pmol of primer, and then DNA synthesis with dideoxynucleotides, ^{35}S-dATP and recombinant DNA polymerase (Sequenase kit; U.S. Biochemical Corp.), followed by polyacrylamide gel electrophoresis (26). Direct sequencing of the smaller

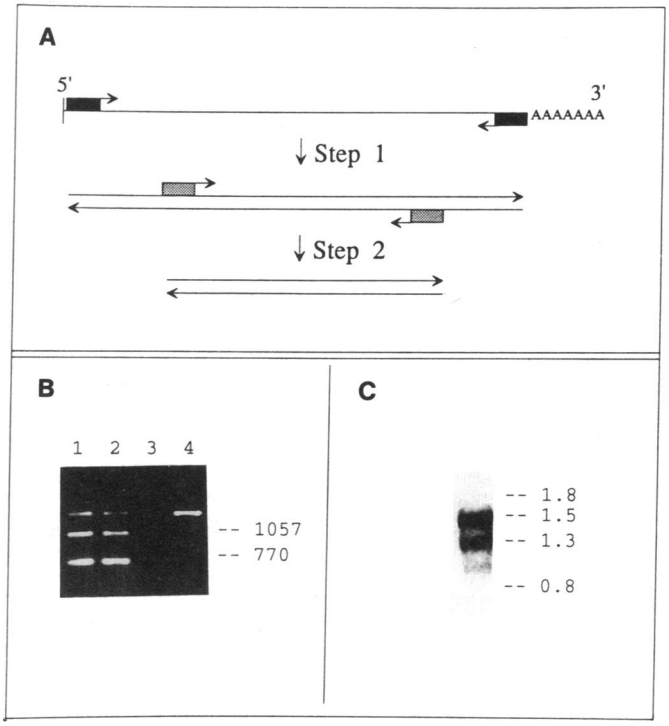

Figure 1. Identification of hFcγRI transcripts of three distinct sizes. (*A*) PCR amplification strategy, schematic diagram. cDNA was synthesized using reverse transcriptase, followed by 30 cycles of PCR using human FcγRI specific primers (shown as dark rectangles). In step 2, a second set of hFcγRI specific primers 30 bp inside of the initial primers (shaded rectangles) was used in a "nested" supplemental 20 cycles of PCR. (*B*) PCR-amplified cDNA, separated by DNA gel electrophoresis. Amplification of hFcγRI cDNA clone p135 (lane *4*) (20, 21) resulted in a single 1,200-bp product, as predicted. Amplification of cDNA from human mononuclear cells (lane *1*) or U937 cells (lane *2*) led to products of 1,200, 1,000, and 750 bp. Without the addition of mRNA (lane *3*), no product was observed. DNA size standards, in basepairs, are shown to the right. (*C*) Northern hybridization of IFN-γ–stimulated U937 RNA with a hFcγRI specific probe also showed three distinct transcripts. Strong bands were identified at 1,500 and 1,250 bp, and a weaker band was seen at 1,000 bp. RNA size standards in kilob are shown to the right.

PCR amplified products was performed using the same technique after purification over Sepharose CL-4B columns (Boehringer Mannheim Biochemicals).

Northern hybridization. 15 μg of RNA was separated on 1.4% agarose containing 2.2 M formaldehyde by electrophoresis at 120 V for 3 h, and blotted onto a 0.45-μm nylon membrane (Schleicher & Schuell, Inc., Keene, NH). After ultraviolet crosslinking, the nylon was prehybridized in 50% formamide containing 100 μg/ml denatured salmon sperm DNA and yeast tRNA at 62°C for 18 h.

A [^{32}P]UTP (NEN, Wilmington, DE) labeled RNA probe was synthesized using SP6 RNA polymerase (Promega, Madison, WI) (27) from the pRc/CMV construct containing the smallest FcγRI (750 bp) PCR product. 10^6 cpm/ml of probe was added to the prehybridization solution, and hybridization was performed for 24 h at 62°C. Hybridized blots were washed at 60°C.

Transfection of COS-7 cells. 10 μg of DNA was precipitated onto a 40% confluent monolayer of COS-7 cells using calcium phosphate (28). After 4 h, the cells were glycerol shocked, and exposed to 100 μM chloroquine for 3 h. After 48 h of culture, the cells were harvested by vigorous aspiration.

Flow cytometry. 2×10^5 cells in 0.05 ml were incubated 30 min at room temperature with first antibody (saturating doses of mouse mono-

A

```
1   ATGTGGTTCTTGACAACTCTGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATC  hFcγRIa1
    ATGTGGTTCTTGACAACTCTGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATC  hFcγRIb1
    ATGTGGTTCTTGACAACTCTGCTCCTTTGGG----------------------------------------  hFcγRIb2

73  ACTTTGCAGCCTCCATGGGTCAGCGTGTTCCAAGAGGAAACCGTAACCTTGCACTGTGAGGTGCTCCATCTG  hFcγRIa1
    ACTTTGCAGCCTCCATGGGTCAGCGTGTTCCAAGAGGAAACCGTAACCTTGCACTGTGAGGTGCTCCATCTG  hFcγRIb1
    ----------------------------------------------------------------------  hFcγRIb2

145 CCTGGGAGCAGCTCTACACAGTGGTTTCTCAATGGCACAGCCACTCAGACCTCGACCCCCAGCTACAGAAT  hFcγRIa1
    CCTGGGAGCAGCTCCACACAGTGGTTTCTCAATGGCACAGCCACTCAGACCTCGACCCCCAGCTACAGAAT  hFcγRIb1
    ----------------------------------------------------------------------  hFcγRIb2

216 CACCTCTGCCAGTGTCAATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCA  hFcγRIa1
    CACCTCTGCCAGTGTCAATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCA  hFcγRIb1
    ----------------------------------------------------------------------  hFcγRIb2

289 TACAGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCACGGAAGGAGAACCT  hFcγRIa1
    TACAGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCATGGAAGGAGAACCT  hFcγRIb1
    --------------------GCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCATGGAAGGAGAACCT  hFcγRIb2

360 CTGGCCTTGAGGTGTCATGCGTGGAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGC  hFcγRIa1
    CTGGCCTTGAGGTGTCATGCGTGGAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGC  hFcγRIb1
    CTGGCCTTGAGGTGTCATGCGTGGAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGC  hFcγRIb2

431 CTTTAAGTTTTTTCCACTGGAATTCTAACCTCACCATTCTGAAAACCAACATAAGTCACAATGGCACCTACC  hFcγRIa1
    CTTTAAGTTTTTTCCACTGGAATTCTAACCTCACCATTCTGAAAACCAACATAAGTCACAATGGCACCTACC  hFcγRIb1
    CTTTAAGTTCTTCCACTGGAATTCTAACCTCGCCATTCTGAAAACCAACATAAGTCACAATGGCACCTACC  hFcγRIb2

502 ATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCT---GTCACTGTGAAAGAGCTATTT  hFcγRIa1
    ATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCACAATACACTGTGAAA---------  hFcγRIb1
    ATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCACAATACACTGTGAAA---------  hFcγRIb2

570 CCAGCTCCAGTGCTGAATGCATCTGTGACATCCCCACTCCTGGAGGGGAATCTGGTCACCCTGAGCTGTGA  hFcγRIa1
    ----------------------------------------------------------------------  hFcγRIb1
    ----------------------------------------------------------------------  hFcγRIb2

641 AACAAAGTTGCTCTTGCAGAGGCCTGGTTTGCAGCTTTACTTCTCCTTCTACATGGGCAGCAAGACCCTGC  hFcγRIa1
    ----------------------------------------------------------------------  hFcγRIb1
    ----------------------------------------------------------------------  hFcγRIb2

712 GAGGCAGGAACACATCCTCTGAATACCAAATACTAACTGCTAGAAGAGAAGACTCTGGGTTATACTGGTGC  hFcγRIa1
    ----------------------------------------------------------------------  hFcγRIb1
    ----------------------------------------------------------------------  hFcγRIb2

783 GAGGCTGCCACAGAGGATGGAAATGTCCTTAAGCGCAGCCCTGAGTTGGAGCTTCAAGTGCTTGGCCTCC  hFcγRIa1
    ----------------------------------------------------------GGCCTCC  hFcγRIb1
    ----------------------------------------------------------GGCCTCC  hFcγRIb2

853 AGTTACCAACTCCTGTCTGGTTTCATGTCCTTTTCTATCTGGCAGTGGGAATAATGTTTTTAGTGAACAC  hFcγRIa1
    AGTTACCAACTCCTGTCTGGTTTCATGTCCTTTTCTATCTGGCAGTGGGAATAATGTTTTTAGTGAACAC  hFcγRIb1
    AGTTACCAACTCCTGTCTGGTTTCATGTCCTTTTCTATCTGGCAGTGGGAATAATGTTTTTAGTGAACAC  hFcγRIb2

923 TGTTCTCTGGGTGACAATACGTAAAGAACTGAAAAGAAAGAAAAAGTGGGATTTAGAAATCTCTTTGGAT  hFcγRIa1
    TGTTCTCTGGGTGACAATACGTAAAGAACTGAAAAGAAAGAAAAAGTGGAATTTAGAAATCTCTTTGGAT  hFcγRIb1
    TGTTCTCTGGGTGACAATACGTAAAGAACTGAAAAGAAAGAAAAAGTGGAATTTAGAAATCTCTTTGGAT  hFcγRIb2

993 TCTGGTCATGAGAAGAAGGTAATTTCCAGCCTTCAAGAAGACAGACATTTAGAAGAAGAGCTGAAATGTC  hFcγRIa1
    TCTGGTCATGAGAAGAAGGTAATTTCCAGCCTTCAAGAAGACAGACATTTAGAAGAAGAGCTGAAATGTC  hFcγRIb1
    TCTGGTCATGAGAAGAAGGTAATTTCCAGCCTTCAAGAAGACAGACATTTAGAAGAAGAGCTGAAATGTC  hFcγRIb2

1063 AGGAACAAAAAGAAGAACAGCTGCAGGAAGGGGTGCACCGGAAGGAGCCCCAGGGGGCCACGTAGCAG  hFcγRIa1
     AGGAACAAAAAGAAGAACAGCTGCAGGAAGGGGTGCACCGGAAGGAGCCCCAGGGGGCCACGTAGCAG  hFcγRIb1
     AGGAACAAAAAGAAGAACAGCTGCAGGAAGGGGTGCACCGGAAGGAGCCCCAGGGGGCCACGTAGCAG  hFcγRIb2
```

Figure 2. Sequences of three hFcγRI transcripts. (*A*) Aligned nucleotide sequences of the three PCR-amplified hFcγRI cDNAs (hFcγRIa1, hFcγRIb1, and hFcγRIb2). Nucleotide differences between hFcγRIa1 and p135 are overlined. Nucleotide differences between hFcγRIa1 and hFcγRIb1,2 (*shadowed*). Nucleotide differences between hFcγRIb1 and hFcγRIb2, (*underlined*). (*B*) Predicted amino acid sequences of the three PCR amplified hFcγRI cDNAs. Amino acid substitutions between hFcγRIa1 and p135, (*overlined*). Amino acid differences between hFcγRIa1 and hFcγRIb1,2, (*shadowed*). Amino acid differences between hFcγRIb1 and hFcγRIb2, (*underlined*). GenBank accession numbers L03418, L03419, and L03420.

B

```
hFcγRIa1  - MWFLTTLLLWVPVDGQVDTTKAVITLQPPWVSVFQEETVTLHCEVLHLPG -50
hFcγRIb1  - MWFLTTLLLWVPVDGQVDTTKAVITLQPPWVSVFQEETVTLHCEVLHLPG -50
hFcγRIb2  - MWFLTTLLLW---------------------------------------- -10

hFcγRIa1  - SSSTQWFLNGTATQTSTPSYRITSASVNDSGEYRCQRGLSGRSDPIQLEI -100
hFcγRIb1  - SSSTQWFLNGTATQTSTPSYRITSASVNDSGEYRCQRGLSGRSDPIQLEI -100
hFcγRIb2  - ---------------------------------------------------- -10

hFcγRIa1  - HRGWLLLQVSSRVFTEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWN -150
hFcγRIb1  - HRGWLLLQVSSRVFMEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWN -150
hFcγRIb2  - --GWLLLQVSSRVFMEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWN -58

hFcγRIa1  - SNLTILKTNISHNGTYHCSGMGKHRYTSAGIS-VTVKELFPAPVLNASVT -199
hFcγRIb1  - SNLTILKTNISHNGTYHCSGMGKHRYTSAGISQKTVK------------- -187
hFcγRIb2  - SNLAILKTNISHNGTYHCSGMGKHRYTSAGISQKTVK------------- -95

hFcγRIa1  - SPLLEGNLVTLSCETKLLLQRPGLQLYFSFYMGSKTLRGRNTSSEYQILT -249
hFcγRIb1  - ---------------------------------------------------- -187
hFcγRIb2  - ---------------------------------------------------- -95

hFcγRIa1  - ARREDSGLYWCEAATEDGNVLKRSPELELQVLGLQLPTPVWFHVLFYLAV -299
hFcγRIb1  - --------------------------------GLQLPTPVWFHVLFYLAV -205
hFcγRIb2  - --------------------------------GLQLPTPVWFHVLFYLAV -113

hFcγRIa1  - GIMFLVNTVLWVTIRKELKRKKKWDLEISLDSGHEKKVISSLQEDRHLEE -349
hFcγRIb1  - GIMFLVNTVLWVTIRKELKRKKKWNLEISLDSGHEKKVISSLQEDRHLEE -255
hFcγRIb2  - GIMFLVNTVLWVTIRKELKRKKKWNLEISLDSGHEKKVISSLQEDRHLEE -163

hFcγRIa1  - ELKCQEQKEEQLQEGVHRKEPQGAT -374
hFcγRIb1  - ELKCQEQKEEQLQEGVHRKEPQGAT -280
hFcγRIb2  - ELKCQEQKEEQLQEGVHRKEPQGAT -188
```

Figure 2 (Continued)

clonal, or dilutions of aggregated human IgG prepared by heat aggregating human Cohn fraction II (Miles Labs, Kanakee, IL) at 63°C for 20 min) in PBS and 0.1% BSA and then washed twice. Cells were subsequently incubated with fluorochrome conjugated $F(ab)'_2$ second antibody (goat anti-mouse or goat anti-human) (Tago, Burlingame, CA) at 4°C for 30 min and washed twice again before flow cytometry with a dual laser Cytofluorograf IIS with a 2151 computer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Preparation of IgG-opsonized erythrocytes. Erythrocytes were coupled to human IgG (E-hIgG) by a biotin-avidin technique, as described previously (29). 5×10^8 erythrocytes were incubated with sulfo-*N*-hydroxysuccinimide-biotin (Pierce Chemical Co., Rockford, IL) (500 μg/ml) for 20 min at 4°C, followed by three washes. Cells at 10^9/ml were mixed with an equal volume of streptavidin (250 μg/ml) for 30 min at 4°C. The streptavidin coated cells (E_{BA}) were then washed and resuspended at 10^9 E/ml for immediate use.

Human IgG was biotinylated with *N*-hydroxysuccinimide-long chain-biotin (0.01 mg biotin/mg protein) for 60 min at room temperature. To bind the biotinylated hIgG to the E_{BA}, E_{BA} (12.5 μl at 10^9/ml) were combined with biotinylated IgG (20 μg) for 30 min at 4°C with mixing. After three washes, the human IgG–coated E_{BA} (E-hIgG) were resuspended in 125 μl (10^8 E/ml) and used immediately.

Alternatively, antibody-coated erythrocytes (EA) were prepared by incubating bovine E with a subagglutinating titer of rabbit IgG anti–bovine E antibody (Cappel Laboratories, Cochranville, PA) for 1 h at 37°C. The cells were washed and resuspended at 10^8 cells/ml in RPMI and 20% FCS.

Rosette formation with IgG-opsonized erythrocytes. COS-7 cells were resuspended in RPMI and 20% heat-inactivated IgG free FCS at 5 $\times 10^6$ cells/ml, as described previously (30). Cells (100 μl) were combined with EA or E-hIgG (250 μl), centrifuged at 150 *g* for 3 min, incubated at 37°C for 1 h, and then gently resuspended. Adherence of EA or E-hIgG to COS-7 cells was assessed by light microscopy.

Results

Amplification of FcγRI transcripts. Using oligonucleotide primers complementary to a known hFcγRI cDNA (20), RT-PCR amplification of RNA extracted from U937 cells and from resting human mononuclear cells of two healthy donors produced little or no product after 30 cycles of amplification. This result probably reflects low hFcγRI mRNA levels in resting cells. To increase sensitivity, a nested PCR strategy was used (Fig. 1 *A*). This technique, with both U937 cell RNA and human mononuclear cell RNA, consistently resulted in three distinct amplified DNA products (Fig. 1 *B*). The longest of these three products, 1,200 bp, was identical in size to the single product resulting from PCR amplification of p135 cDNA. The two smaller amplified DNA products were ~ 1,000 and 750 bp in length.

Northern hybridization. To confirm the existence of these smaller hFcγRI related transcripts, nonamplified RNA from U937 cells was analyzed on Northern blots. To enhance the ability to detect low levels of message, a ^{32}P-labeled antisense RNA probe with high avidity for mRNA was used instead of a cDNA probe. IFN-γ–treated U937 cells showed transcripts of

1,500 and 1,250 bp, with a weaker third transcript at 1,000 bp (Fig. 1 *C*). These messages most likely correspond to the 1,200-, 1,000-, and 750-bp PCR amplified cDNAs, respectively.

Characterization of PCR amplified transcripts. To characterize each amplified cDNA, each product was cloned into vector pRc/CMV and sequenced by the dideoxy technique. The coding cDNA and predicted protein sequences of these three products are shown in Fig. 2.

The 1,200-bp PCR amplified cDNA is similar but not identical to previously described hFcγRI gene transcripts described by Allen and Seed (20). It differs at nucleotide positions 73 and 1,024 from the p135 cDNA clone (20), resulting in nonconservative amino acid substitutions at positions 25 and 338. However, the coding sequence exactly matches the nucleotide and predicted protein sequences described for the FcγRIA gene by van de Winkel (18). Direct sequencing of all 1,200-bp PCR products from two healthy individuals showed both individuals to be homozygous for the same hFcγRIA sequence.

The 1,000- and 750-bp cDNA sequences both lack the exon encoding EC3, and the 750-bp cDNA additionally lacks the exon encoding EC1. Both of these transcripts also have a series of seven consecutive divergent nucleotides (nucleotide positions 544–550), which result in an inserted amino acid and a nonconservative amino acid substitution near the end of the exon encoding EC2 (amino acid positions 183–184, FcγRIA = Val, FcγRIB = Gln, Tyr). These amino acid substitutions agree with a preliminary, partial sequence of a second hFcγRIB gene reported recently (19), and therefore both the 1,000- and 750-bp cDNAs probably represent transcription products of a hFcγRIB gene. These putative hFcγRIB gene products both differ from the A gene at nucleotide positions 346 and 981 and at predicted amino acid (hFcγRIa1) positions 115 (FcγRIA = Thr, FcγRIB = Met) and 324 (FcγRIA = Asp, FcγRIB = Asn). In addition to the presence or absence of the exon encoding EC1, which is presumably caused by alternative splicing, the two hFcγRIB gene product sequences also differ from each other at nucleotides 440 (silent) and 462, the latter predicting an amino acid substitution at position 154 in the larger gene product.

Using primers complementary to both hFcγRIA and hFcγRIB, direct sequencing of 1,200-bp PCR amplified cDNA from mononuclear cells of two individuals showed no evidence of a B gene transcription product that would include the exon encoding EC3 and lead to a full-length B gene product with three extracellular immunoglobulin-like domains.

Expression of Cloned FcγRI Transcripts. COS-7 cells transiently transfected with pRc/CMV vector constructs containing hFcγRIA cDNA express a surface protein recognized by FcγRI-specific mAbs (10.1, 22.2, 32.2, 44, 62, and 197) as measured by flow cytometry (Fig. 3 *A*). These cells bind both monomeric and heat-aggregated human and murine IgG (Fig. 3 *C*), and form rosettes with E-hIgG (Fig. 4 *A*). Therefore, the transfected full-length hFcγRIA cDNA encodes a surface molecule with binding properties and epitopes matching those of the high affinity IgG receptor.

COS-7 cells transfected with plasmid containing the larger hFcγRIB cDNA have very different properties. These cells are not recognized by any of the above FcγRI-specific mAbs (Fig. 3 *B*), nor by several mAbs specific for FcγRII (IV.3, 41H16) and FcγRIII (3G8). These cells have no appreciable binding of monomeric IgG, as measured by flow cytometry.

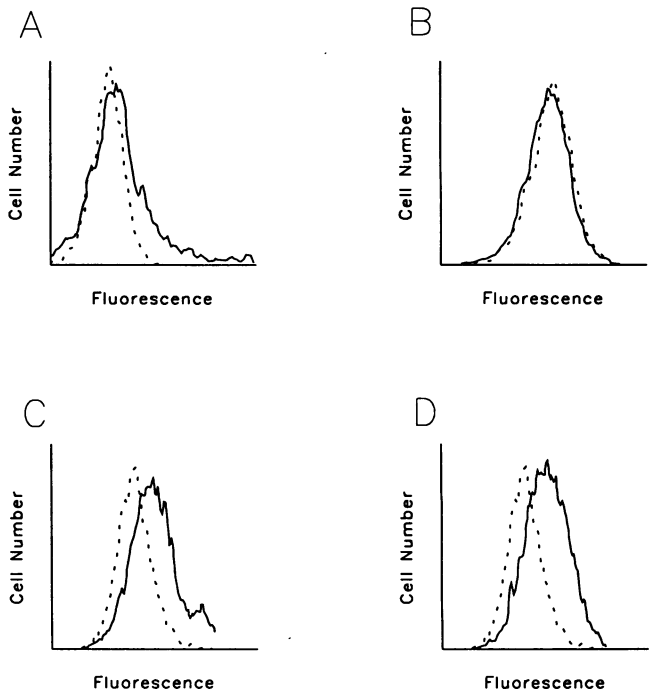

Figure 3. Binding properties of COS-7 cells transfected with hFcγRI isoforms. (*A–D*) Indirect immunofluorescence of transfected cells, as measured by flow cytometry. Cells transfected with plasmid alone are shown as a dashed line. Fluorescence intensity is plotted on a logarithmic scale. (*A, C*) Cells transfected with plasmid containing FcγRIa1 cDNA are shown as a solid line. (*B, D*) Cells transfected with plasmid containing FcγRIb1 cDNA are shown as a solid line. (*A, B*) Staining with mAb 32.2. Results with mAbs 10.1, 22.2, 32.2, and 197 were similar. Cells transfected with hFcγRIa1 are recognized by FcγRI specific monoclonal antibodies (*A*), but cells receiving hFcγRIb1 cDNA were not recognized (*B*). (*C, D*) Staining with heat-aggregated human IgG. Cells transfected with either hFcγRIa1 (*C*) or hFcγRIb1 (*D*) bind heat-aggregated human IgG.

However, expression of the larger hFcγRIB cDNA on COS-7 cells did result in binding of multimeric IgG. hFcγRIB-transfected cells bind E-hIgG and reproducibly form rosettes, although with lower efficiency compared with cells transfected with FcγRIA (Fig. 4 *B*). Results with EA, using rabbit IgG, were similar. Furthermore, cells expressing the larger hFcγRIB are able to bind heat-aggregated human IgG, as measured by flow cytometry (Fig. 3 *D*). Interestingly, no measurable binding of murine heat-aggregated murine IgG could be detected, suggesting that binding may show species specificity.

Regulation of message levels by IFN-γ. Since the FcγRIA gene mRNA level and protein product can be induced by IFN-γ, we sought evidence that transcripts for the two alternative splice products of the putative FcγRIB are similarly regulated by performing Northern hybridizations using a ^{32}P-labeled RNA probe. Unstimulated human mononuclear cells and U937 cells show low levels of at least two transcripts, one of 1,500 and one of 1,250 bp (Fig. 5). The 1,250-bp product is less prominent in human mononuclear cells, while unstimulated human neutrophils have no detectable FcγRI related transcripts. Treatment of all cell types with recombinant human IFN-γ markedly increases levels of FcγRI transcripts. In both human mononuclear cells and U937 cells, an increase in the level of both 1,500- and 1,250-bp transcripts was seen, al-

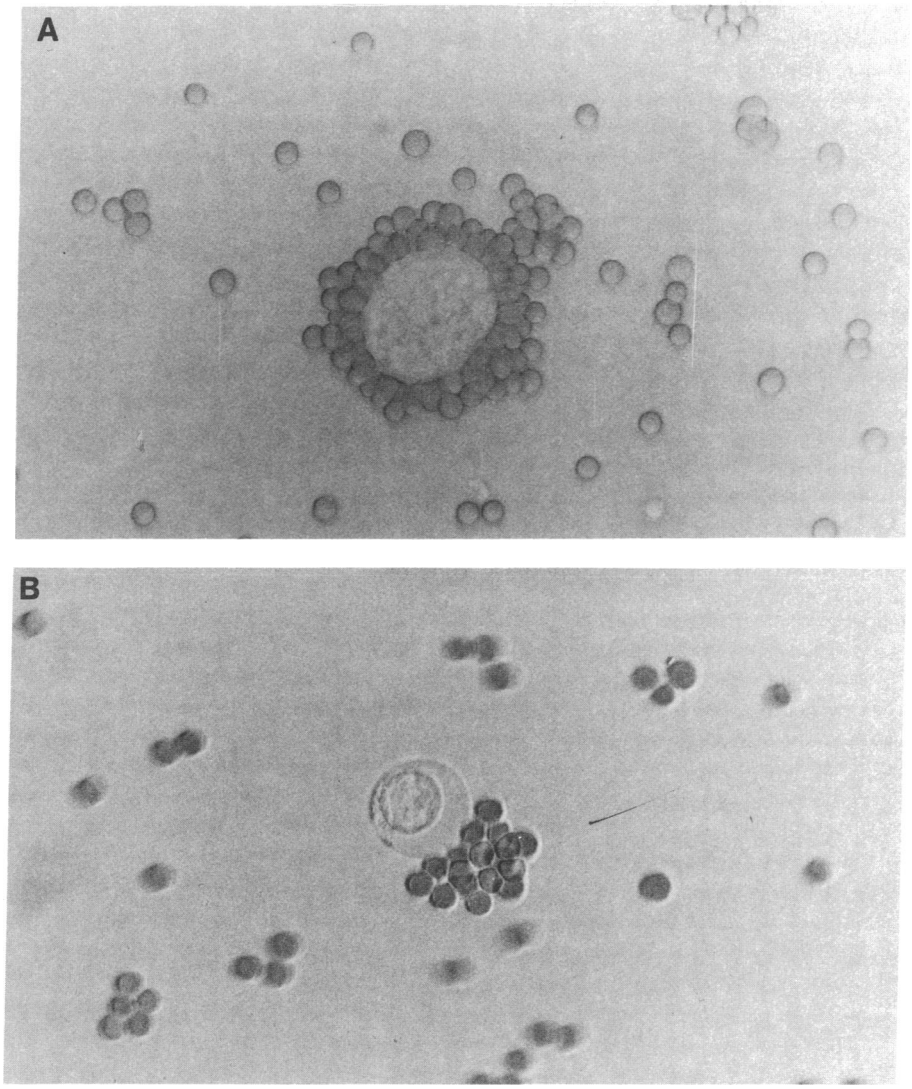

Figure 4. Rosetting patterns of transfected COS-7 cells. Erythrocytes are coated with human IgG via a biotin-avidin linkage. (*A*) COS-7 cells transfected with hFcγRIa1. Rosetting of IgG coated RBCs is observed. (*B*) COS-7 cells transfected with hFcγRIb1. A less vigorous rosetting pattern can be observed with transfection of the hFcγRIb1 cDNA.

though the smaller band was less enriched by IFN-γ stimulation in mononuclear cells. In the case of U937 cells, a third 1,000-bp transcript could also be clearly identified. For IFN-γ treated neutrophils, enrichment of all three transcription products was even more pronounced.

Discussion

The role of receptors from the FcγRI family in modulating the immune response has been enigmatic, in part because structural diversity seen in other FcγR families (FcγRII and FcγRIII) has not been previously identified in the human FcγRI family. The ability of IFN-γ to regulate quantitative expression of the high affinity FcγRI implies an important role in immune regulation. However, some investigators have argued that the constitutive univalent occupation of the FcγRI ligand binding site precludes its participation in the handling of many immune stimuli, including immune complexes. Much of the debate about the role of the FcγRI family, however, is obviated by this and other (19) demonstrations of multiple transcripts in the hFcγRI family, and by our recognition of both high and low affinity FcγRI cell surface receptors.

Both PCR amplification and Northern hybridizations of nonamplified cellular RNA from human mononuclear cells, the human monocyte-like cell line U937, and human neutrophils have provided evidence for at least three distinct hFcγRI-related transcripts. Direct sequencing of the largest PCR product has demonstrated it to be identical to the full-length sequence of the FcγRIA gene described by van de Winkel (18). This product differs from the p135 cDNA at nucleotide positions 73 and 1,024, resulting in nonconservative amino acid substitutions at positions 25 and 338 (20). These differences in the p135 sequence may represent allelic polymorphisms or amplification artifact. It is interesting to speculate whether the single amino acid difference in EC1 of p135 might explain the lower than expected affinity for monomeric IgG of the p135 product expressed in COS cells (21).

The intermediate-sized PCR product corresponds to the 1,250-bp transcript on our Northern hybridizations of human mononuclear cells, U937 cells, and IFN-γ stimulated neutrophils, and perhaps corresponds to the second transcript band in other Northerns hybridized with hFcγRI (p135) probes (21). This product shows several features that distinguish it from the FcγRIA gene product, and which indicate that it corresponds

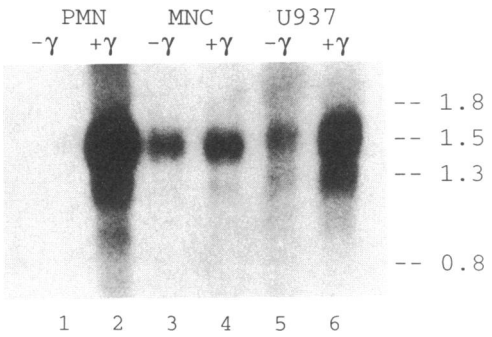

Figure 5. Northern hybridization using a hFcγRI specific probe under high stringency. (Lanes *1, 2*) Neutrophil RNA. (Lanes *3, 4*) Mixed mononuclear cell RNA. (Lanes *5, 6*) U937 cell RNA. (Lanes *2, 4, 6*) IFN-γ (200 U/ml) added for 6 h before RNA extraction. Molecular weight standards are shown on the right. Neutrophils cultured without IFN-γ showed no FcγRI related transcripts, but IFN-γ inducible bands can be seen at 1,500, 1,250, and 1,000 bp. Cultured mononuclear cells show a 1,500-bp band, and a weakly IFN-γ inducible 1,000-bp band. The unstimulated monocytoid cell line U937 demonstrates 1,500 and 1,250 bp transcripts, with enhancement of 1,500, 1,250, and 1,000 bp transcripts after culture with IFN-γ.

to a product of the FcγRIB gene (18, 19). Two nonconservative amino acid substitutions found in this product at the end of the exon encoding EC2 are characteristic of the hFcγRIB gene. Interestingly, this transcript, named hFcγRIb1, lacks the entire exon encoding EC3. Since this exon in the hFcγRIB gene contains a stop codon (19), the absence of this exon at the message levels allows the translation of a mature protein with both a transmembrane and a cytoplasmic domain. This protein can be expressed in COS-7 cells. Much like the murine FcγRI chimera, which contained EC1 and EC2 but not EC3 and had the properties of a low affinity receptor (12), hFcγRIb1 does not bind monomeric IgG but does have the capacity to bind human IgG aggregates.

The transcript on Northern hybridization corresponding to the smallest PCR product was not easily visualized in resting mononuclear and U937 cells, but like the two larger products it was IFN-γ inducible, especially in human neutrophils. Sequence analysis showed that this product has nucleotide substitutions matching the FcγRIB gene (18, 19), but unlike the FcγRIb1 product, this transcript (FcγRIb2) lacks both EC1 and EC3. Clones of hFcγRIb2 also have two nucleotide (and one amino acid) substitutions relative to hFcγRIb1 clones. While both of these transcripts probably arise through alternative splicing of hFcγRIB mRNA, we cannot exclude the possibility that the "hFcγRIb2" transcript results from a distinct ("hFcγRIC") gene. Alternatively, the two nucleotide substitutions may represent allelic polymorphisms, or perhaps cloning artifact. The properties of the expressed hFcγRIb2 protein are currently unexplored.

COS-7 cells transfected with hFcγRIb1 cDNA confirmed that the encoded protein has binding properties analogous to other low affinity receptors for human IgG. hFcγRIb1-transfected COS-7 cells formed rosettes with antibody-coated erythrocytes, although more weakly than cells expressing hFcγRIa1. hFcγRIb1-transfected COS-7 cells were unable to bind monomeric IgG, but were able to bind heat-aggregated human IgG. Thus, hFcγRIb1 has the capacity to recognize immune complexes. The hFcγRI family is therefore unique in having both high and low affinity members.

The identification of hFcγRIb1 and hFcγRIb2 demonstrates that the hFcγRI family has significant structural diversity, both at the genomic and mRNA levels. The FcγRII and FcγRIII families also demonstrate extensive structural diversity. Results from Northern hybridization confirm that each of these hFcγRI transcripts is IFN-γ inducible. Given the distinct cytoplasmic domain of the FcγRI family members, one can anticipate unique roles for hFcγRIb1, hFcγRIb2, and probably other unrecognized members of the hFcγRI family in the humoral immune response.

Acknowledgments

The authors thank Nina Brogle and Carl Triscari for expert technical assistance.

Dr. Porges is a recipient of an Arthritis Foundation Postdoctoral Fellowship. This work was supported by a grant from the Systemic Lupus Erythematosus (S.L.E.) Foundation of New York City.

References

1. Anderson, C. L., and R. J. Looney. 1986. Human leukocyte IgG Fc receptors. *Immunol. Today.* 7:264–266.

2. Unkeless, J. C. 1989. Function and heterogeneity of human Fcγ receptors for immunoglobulin G. *J. Clin. Invest.* 83:355–361.

3. Unkeless, J. C., E. Scigliano, and V. H. Freedman. 1988. Structure and function of human and murine receptors for IgG. *Annu. Rev. Immunol.* 6:251–281.

4. Ravetch, J. V., and C. L. Anderson. 1990. FcγR family: proteins, transcripts, and genes. *In* Fc Receptors and the Action of Antibodies. H. Metzger, editor. American Society for Microbiology, Washington, DC. 211–235.

5. van de Winkel J. G. J., and C. L. Anderson. 1991. Biology of human immunoglobulin G Fcγ receptors. *J. Leukocyte Biol.* 49:511–524.

6. Brooks, D. G., W. Q. Qiu, A. D. Luster, and J. V. Ravetch. 1989. Structure and expression of human IgG FcγRII. Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *J. Exp. Med.* 170:1369–1385.

7. Ravetch, J. V., and B. Perussia. 1989. Alternative membrane forms of FcγRIII on human natural killer cells and neutrophils. *J. Exp. Med.* 170:481–497.

8. Qiu, W. Q., D. deBruin, B. H. Brownstein, R. Pearse, and J. V. Ravetch. 1990. Organization of the human and mouse low-affinity FcγR genes: duplication and recombination. *Science (Wash. DC).* 248:732–735.

9. Anderson, C. L. 1982. Isolation of the receptor for IgG from a human monocyte cell line (U937) and from human peripheral blood monocytes. *J. Exp. Med.* 156:1794–1805.

10. Dougherty, G. J., Y. Selvendran, S. Murdoch, D. G. Palmer, and N. Hogg. 1987. The human mononuclear phagocyte high-affinity Fc receptor, FcγRI, defined by a monoclonal antibody, 10.1. *Eur. J. Immunol.* 17:1453–1459.

11. Peltz, G., K. Frederick, C. L. Anderson, and B. M. Peterlin. 1988. Characterization of the human monocyte high affinity Fc receptor. *Mol. Immunol.* 25:243–250.

12. Hulett, M. D., N. Osman, I. F. McKenzie, and P. M. Hogarth. 1991. Chimeric Fc receptors identify functional domains of the murine high affinity receptor for IgG. *J. Immunol.* 147:1863–1868.

13. Ohta, Y., T. P. Stossel, and J. H. Hartwig. 1991. Ligand-sensitive binding of actin-binding protein to immunoglobulin G Fc receptor (FcγRI). *Cell.* 67:275–282.

14. Perussia, B., E. T. Dayton, R. Lazarus, V. Fanning, and G. Trinchieri. 1983. Immune interferon induces the receptor for monomeric IgG on human monocytic and myeloid cells. *J. Exp. Med.* 158:1092–1113.

15. Petroni, K. C., L. Shen, and P. M. Guyre. 1988. Modulation of human polymorphonuclear leukocytes IgG Fc receptors and Fc receptor mediated function by IFN-γ and glucocorticoids. *J. Immunol.* 140:3467–3472.

16. Pan, L., D. B. Mendel, J. Zurlo, and P. M. Guyre. 1990. Regulation of the steady state of FcγRI mRNA by IFN-γ and dexamethasone in human monocytes, neutrophils, and U937 cells. *J. Immunol.* 145:267–275.

17. Pearse, R. N., R. Feinman, and J. V. Ravetch. 1991. Characterization of the promoter of the human gene encoding the high-affinity IgG receptor: transcriptional induction by γ-interferon is mediated through common DNA response elements. *Proc. Natl. Acad. Sci. USA.* 88:11305–11309.

18. van de Winkel, J. G. J., L. K. Ernst, C. L. Anderson, and I. M. Chiu. 1991. Gene organization of the human high affinity receptor for IgG, FcγRI. *J. Biol. Chem.* 286:13449–13455.

19. Ernst, L. K., J. G. J. van de Winkel, I. M. Chiu, and C. L. Anderson. 1992. Three genes for the human high affinity Fc receptor for IgG encode four distinct transcription products. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:A2011. (Abstr.)

20. Allen, J. M., and B. Seed. 1988. Nucleotide sequence of three cDNAs for the human high affinity Fc receptor FcRI. *Nucleic Acids Res.* 16:11824.

21. Allen, J. M., and B. Seed. 1989. Isolation and expression of functional high-affinity Fc receptor complementary DNAs. *Science (Wash. DC).* 243:378–381.

22. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21S:77–89.

23. Strohman, R. C., P. S. Moss, J. Micou-Eastwood, et al. 1977. Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. *Cell.* 10:265–273.

24. Saiki, R. K., D. H. Gelfand, S. Stoffel, et al. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerse. *Science (Wash. DC).* 239:487–491.

25. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1.53–1.85.

26. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

27. Zinn, K., D. DiMaio, and T. Maniatis. 1983. Interaction of two distinct regulatory regions adjacent to the human β-interferon gene. *Cell.* 34:865–879.

28. Parker, B. A., and G. R. Stark. 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. *J. Virol.* 31:360–369.

29. Edberg, J. C., and R. P. Kimberly. 1992. Receptor specific probes for the study of Fcγ receptor specific function. *J. Immunol. Methods.* 148:179–187.

30. Salmon, J. E., J. C. Edberg, and R. P. Kimberly. 1990. Fcγ receptor III on human neutrophils. Allelic variants have functionally distinct capacities. *J. Clin. Invest.* 85:1287–1295.