Recombinant Soluble Human FcγRII: Production, Characterization, and Inhibition of the Arthus Reaction

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Summary

A recombinant soluble form of human FcγRII (rsFcγRII) was genetically engineered by the insertion of a termination codon 5′ of sequences encoding the transmembrane domain of a human FcγRII cDNA. Chinese hamster ovary cells were transfected with the modified cDNA and the secreted rsFcγRII purified from the tissue culture supernatant (to >95%, assessed by SDS-PAGE) using heat aggregated human immunoglobulin G (IgG) immunoaffinity chromatography. The IgG-purified rsFcγRII was relatively homogeneous (~31,000 M_r) whereas the total unpurified rsFcγRII secreted into the tissue culture supernatant was heterogeneous relating to N-linked glycosylation differences. Functional in vitro activity of the rsFcγRII was demonstrated by: (*a*) ability to bind via the Fc portion of human IgG and mouse IgG (IgG2a>IgG1>>IgG2b); (*b*) complete inhibition of binding of erythrocytes sensitized with rabbit IgG to membrane-bound FcγRII on K562 cells; and (*c*) inhibition of the anti-Leu4–induced T cell proliferation assay. Blood clearance and biodistribution studies show the rsFcγRII was excreted predominantly through the kidney in a biphasic manner, with an α-phase ($t1/2$ ~25 min) and a β-phase ($t1/2$ ~4.6 h); the kidneys were the only organs noted with tissue-specific accumulation. In vivo, the administration of rsFcγRII significantly inhibited the immune complex–mediated inflammatory response induced by the reversed passive Arthus reaction model in rats. There was a specific and dose-dependent relationship between the amount of rsFcγRII administered, and the reduction in the size and severity of the macroscopic inflammatory lesion. Histological analysis of the skin showed a diffuse neutrophil infiltrate in both control and rsFcγRII-treated rats, however the perivascular infiltrate and the red cell extravasation was less intense in the rsFcγRII-treated group. It is likely that complement activation leads to neutrophil chemotaxis, but neutrophil activation via FcγRII, which results in inflammatory mediator release, is inhibited. The data indicate that rsFcγRII is a potential therapeutic agent for the treatment of antibody or immune complex–mediated tissue damage.

Fc receptors for IgG (FcγR) exist in two forms: membrane-bound FcγR and soluble FcγR (sFcγR)[1] which circulate in biological fluids. The two forms of FcγRs have been described in both human and murine systems, and have a central role in the regulation of the immune response (1). Membrane-bound human FcγRs are widely expressed on hemopoietic cells and can be divided into three major classes on the basis of their structure and affinity for IgG: FcγRI, FcγRII, and FcγRIII (2, 3). FcγRs are important signal transduction molecules, and the interaction of immune complexes through the Fc portion of IgG or mAbs with cell surface FcγRs triggers the activation of monocytes, neutrophils, and platelets (4–15). The binding of immune-complexes to monocytes and neutrophils stimulates the release of inflammatory mediators such as leukotrienes, prostaglandins, and cytokines (TNF-α, IL-1, and IL-6), inducing tissue damage (16–22). Although the exact role of the FcγR(s) in mediating each of these effects has not been fully determined, human FcγRII has been definitively shown to signal immune complex–mediated activation of neutrophils and monocytes. In addition, platelets interact with immune complexes by cell surface FcγRII (the only FcγR expressed on platelets), leading to the secretion of mediators and platelet aggregation (8–15).

Endogenous and recombinant sFcγR have been shown to have both in vitro and in vivo immunoregulatory activity

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; EA, sheep red blood cell sensitized with rabbit IgG; HAGG, heat aggregated human IgG; MSX, L-methionine sulphoxamine; RPAR, reversed passive Arthus reaction; sFcγR, soluble receptor for the constant region of IgG.

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and functional properties (23–32). In vitro, endogenous sFcγR are released from cells expressing membrane-bound FcγR: activated T cells, B cells, macrophages, monocytes, and granulocytes (33–39). Soluble FcγR can be released by proteolysis of membrane-bound FcγR (28, 29, 33), or possibly by alternative RNA splicing of the transmembrane exon (40), and can be stimulated by Ig and cytokines (1). Endogenous mouse sFcγR has a relative molecular mass of 35–40 kD and reacts with an anti-mouse FcγRII mAb, 2.4G2. This antigenic similarity provides further evidence that endogenous sFcγRII and membrane-bound FcγRII are structurally related (41). In vitro, murine sFcγRII can modulate the immune response by inhibition of four separate pathways: (*a*) binding of immune complexes to cell surface FcγRII; (*b*) C1q binding to IgG; (*c*) B cell growth; and (*d*) IgG and IgM antibody production from B cells (1, 23–29). Endogenous murine sFcγRII can be detected in serum of normal mice, and is elevated in states of altered immunity and tumor-bearing mice, indicating it may have an important biological role in vivo (36, 42–46). Supporting this concept, murine recombinant sFcγRII has been demonstrated to inhibit the in vivo IgG response in mice (29). Similarly in humans, sFγRIII can be detected in vivo from serum of normal individuals and patients with autoimmune diseases, and at sites of inflammation (47–49). The immunosuppressive properties of sFcγRII and its potential ability to inhibit membrane-bound FcγRII-mediated cellular activation by immune complexes, opens new therapeutic pathways for the treatment of antibody or immune complex–mediated tissue injury. This study describes the production, characterization, in vitro and in vivo activity of a human rsFcγRII.

Materials and Methods

Genetic Engineering and Production of rsFcγRII. Using the PCR, cDNA for a truncated soluble form of human FcγRII was produced by inserting a premature termination codon 5′ of the transmembrane domain of a membrane-bound FcγRII cDNA (HFc3.0) (50). The HFc3.0 cDNA encodes the FcγRIIa allelic variant expressing glutamine and histidine at amino acid positions 27 and 131, respectively. The oligonucleotide primers used in the PCR were 5′ oligonucleotide, NR1 (5′TACGAATTCCTATGGAGACCCAAATGTCTC3′), and 3′ oligonucleotide, F12 (5′CATTCTAGACTATTGGACAGTGATGGTCAC3′). Both oligonucleotide primers were phosphorylated (100 ng primer, 2 μl 10 mM ATP, 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, 80 U polynucleotide kinase [Pharmacia, Uppsala, Sweden], in 200 μl, incubated for 1 h at 37°C) and used to amplify the mutated cDNA (100 ng of membrane-bound FcγRII cDNA HFc3.0, 500 ng of primers, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 2.5 U Replinase [Dupont, Boston, MA]) with a 30 cycle PCR. The oligonucleotide F12 converted the Val^{171} codon to a TAG stop codon in the HFc3.0 amplified cDNA, resulting in a 635-bp blunt-ended nucleotide fragment, which was ligated into the SmaI restriction site of the linearized dephosphorylated pEE6/HCMV/GS eukaryotic expression vector (Celltech, Slough, England). The plasmid construct was transfected into Chinese hamster ovary (CHO) cells using the calcium phosphate precipitation method (51, 52) and grown in glutamine-free Glasgow's modified Eagles medium (Flow Laboratories, Melbourne, Australia) with 10% FCS (Commonwealth Serum Laboratories, Melbourne, Australia) dialyzed against PBS. The transfected cells were selected with 15–100 μM L-methionine sulphoxamine (MSX) (Sigma Chemical Co., St. Louis, MO) and the supernatants from surviving clones were screened for the presence of rsFcγRII by their ability to inhibit the binding of an anti-FcγRII mAb, 8.26, to $FcγRII^+$ K562 cells, and an ELISA using two anti-FcγRII mAbs (8.26 and 8.7) (53). The level of rsFcγRII in tissue culture supernatant was increased by: (*a*) gene amplification; clones originally selected at 15 μM MSX were further selected 100 μM MSX; and (*b*) growing the transfected CHO cells in the presence of 2 mM sodium butyrate. For large scale production, transfected cells were grown in roller bottles (850 cm^2, Becton Dickinson & Co., Mountain View, CA) seeded at 5×10^7 cells/ml and grown for 14 d until the cells were detached.

Immunoaffinity Purification of the rsFcγRII. Cell culture supernatant containing the rsFcγRII was passed through a 0.2-μm filter and the sample was loaded onto an affinity column containing heat aggregated human IgG (HAGG) coupled to Sepharose 4B beads (Pharmacia). To produce this column, 10 mg of HAGG per 1 g of CNBr-activated freeze-dried powder was coupled according to the manufacturer's guidelines (Pharmacia). HAGG was prepared by heating 6 mg/ml of human IgG (Sandoz, Melbourne, Australia) to 63°C for 30 min. After washing the column with PBS, the bound rsFcγRII was eluted with a 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl. The eluant was immediately neutralized with saturated Tris-HCl and dialyzed against PBS. The molecular weight and purity of the rsFcγRII were determined by SDS-PAGE analysis. The protein content of the purified samples was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon 1\% = 22$ for a 1 mg/ml protein solution calculated from an amino acid analysis of the rsFcγRII. The purification was carried out at 4°C and the purified protein stored frozen at −70°C in PBS. Samples were examined for the presence of endotoxin using the Multi-Test Limulus Amebocyte Lysate Pyrogent (Whittaker Bioproducts, Walkersville, MD).

Biosynthetic Labeling and Deglycosylation of the rsFcγRII. Deglycosylated recombinant protein was produced by adding 5 μg/ml tunicamycin (Sigma Chemical Co.) to the secreting transfected CHO cells for 2 h at 37°C; control transfected cells were grown in the absence of tunicamycin. Both control and tunicamycin-treated cells were pulsed for 2 h with 0.5 mCi of [^{35}S]methionine and [^{35}S]cysteine (Amersham International, Amersham, UK) in glutamine/methionine/cysteine free medium (Select-Amine; GIBCO BRL, Gaithersburg, MD) followed by a 2-h incubation in the presence of 100-fold excess cold methionine/cysteine. Metabolically labeled supernatants from tunicamycin-treated and untreated transfected cells were precleared for 30 min at 4°C with packed Sepharose 4B beads coupled to protein A (Pharmacia) and immunoprecipitation (using 1 ml of radiolabeled supernatant per mAb) carried out for 1 h at 4°C with 30 μl of packed Sepharose 4B beads conjugated to the following mAbs: (*a*) anti-FcγRII mAbs, 8.26 F(ab′), 8.2 $F(ab')_2$, 8.7 $F(ab')_2$, IV.3 F(ab′), and CIKM5 $F(ab')_2$, and a control mAb, 1705 (5084–4.1) $F(ab')_2$, were used with supernatant from transfected cells grown in the absence of tunicamycin; and (*b*) mAb 8.26 was used to immunoprecipitate from supernatant harvested from transfected CHO cells treated with tunicamycin (53–56). The beads were washed with a buffer containing PBS, 1% BSA, 1 mM PMSF, and 0.1% vol/vol aprotinin, pH 7.4 (Sigma Chemical Co.), placed in 20 μl of SDS-PAGE sample buffer (0.1 M Tris-HCl, pH 7.5, 0.1% SDS, 0.1 M dithiothreitol), boiled for 5 min, analyzed by SDS-PAGE on a 13% gel which was dried and autoradiographed.

Immunoprecipitation and SDS-PAGE Analysis. Purified rsFcγRII (100 μg) was radiolabeled with ^{125}I (Amersham International) using chloramine T (57). The rsFcγRII was diluted to 0.2 μg/ml in a buffer containing PBS, 1% BSA, 1 mM PMSF, and 0.1% vol/vol aprotinin, pH 7.4, precleared, and immunoprecipitation (0.2 μg of labeled rsFcγRII per antibody) was carried out as described above with 30 μl of packed Sepharose 4B beads conjugated to the following antibodies: (*a*) anti-human FcγRII mAb, 8.26 F(ab'); (*b*) control antibody, 1705 $F(ab')_2$, mouse IgG2a anti-Ly-12.1; (*c*) whole mouse IgG1, l-1 anti-CEA; (*d*) whole mouse IgG2a 1302 (49-11.1), anti-Ly-2.1; (*e*) whole mouse IgG2b 1480 (5041-24.2), anti-Ly-6A.2; (*f*) whole mouse IgG3, 1308 (49-31.1), anti-Ly-2.1; and (*g*) HAGG (53, 56, 58-60).

Erythrocytes Sensitized with Rabbit IgG (EAs) Rosetting Inhibition Assays. The ability of the rsFcγRII to block the binding immune complexes to membrane-bound FcγRII was determined by the inhibition of EA rosette formation. Starting at a final concentration of 0.4 mg/ml, doubling dilutions of the purified rsFcγRII or a control protein, OVA, were incubated with 50 μl of freshly prepared 2% EA (rabbit anti-sheep red cell polyclonal antibody diluted 1:50 bound to sheep red cells) for 1 h on ice (61). K562 cells (25 μl at 5 × 10^6 cells/ml) expressing FcγRII were added to the EAs and rsFcγRII, incubated for 5 min at 37°C, spun at 200 *g* for 3 min, and the pelleted cells incubated for 30 min on ice. Cells were stained with 0.1% ethyl violet and a typical field of 100 cells was assessed for rosette formation (at least five red cells or 50% of the target cell covered).

Inhibition of the Anti-Leu4-induced T Cell Proliferation Assay by rsFcγRII. In this assay, T cells coated with anti-CD3 antibody can be cross-linked via the Fc portion, by the FcγR on monocytes (62, 63). This phenomenon can be inhibited by rsFγRII. PBMC from an individual were isolated using a Ficoll density gradient (Pharmacia), harvested, washed, and resuspended in RPMI 1640 (Flow Laboratories) supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Commonwealth Serum Laboratories) and 0.05 mM 2-ME (Kock-Light Ltd., Suffolk, UK) at 5 × 10^6 cells/ml. Cells (100 μl vol) were aliquoted into U-bottomed sterile 96-well microtiter plates (Nunc, Roskilde, Denmark) and incubated with 50 μl of anti-Leu4 (Becton Dickinson & Co., Mountain View, CA) at 1:1,000 dilution and 50 μl of serially diluted rsFcγRII or control protein, OVA, starting at 1.0 mg/ml final concentration. Incubation was carried out for 72 h at 37°C, and over the last 14 h cells were pulsed with 1 μCi/well [^3H]thymidine (3,000 Ci/mol, Amersham International). Cells were harvested onto glass paper discs and incorporation of [^3H]thymidine measured by liquid scintillation counting. It should be noted that the anti-CD3 (murine IgG1)-induced T cell response is characterized by a polymorphism with high and low responses (62, 63). A high response individual was used in this study.

Blood Clearance and Biodistribution Studies in Mice. BALB/c mice were used to estimate the in vivo half-life of the rsFcγRII and the tissue distribution after an intravenous dose. Purified rsFcγRII (100 μg) was radiolabeled with ^{125}I using chloramine T (57) and free ^{125}I removed using PD-10 sephadex column (Pharmacia) (<5% free ^{125}I was present as shown by TCA protein precipitation). Groups of four mice were injected intravenously with radiolabeled rsFcγRII and were subsequently killed at various time points collecting blood, urine, and tissues; each sample was measured for radioactivity expressed as cpm. For the blood clearance study, the blood volume was calculated as 7% of the body weight and the half-life of the rsFcγRII obtained from a logarithmic plot of percent injected dose versus time, where $t1/2 = \ln 2/\text{gradient}$ (64). Radioactivity in the urine was expressed as percent injected dose/ml of urine, and the organs as percent injected dose/gram of tissue.

Arthus Reaction. A modified reversed passive Arthus reaction (RPAR) model (65) was established using 4–6-wk-old Sprague-Dawley rats (Austin Research Institute) anesthetized with an i.p. injection of 1.5–2.0 ml of a 1.9% 2,2,2-tribromoethanol (Aldrich Chemical Co., Milwaukee, WI) solution. The rats were shaved, and 5 min after a 5 mg i.v. injection of OVA into the tail vein, the back of the rat was injected intradermally with either: (*a*) 50 μl of purified rabbit IgG anti-OVA (500 μg) with 50 μl of PBS, positive control (after titration, limiting amounts of rabbit IgG anti-OVA were used to give maximal inhibition, similar to that used elsewhere) (66); (*b*) 50 μl of purified nonimmune rabbit IgG (500 μg) with 50 μl of PBS, specificity control; (*c*) 50 μl of rabbit IgG anti-OVA (500 μg) with 50 μl of rsFcγRII at varying doses (50–500 μg) or control protein KLH (500 μg) (Sigma Chemical Co.); or (*d*) rsFcγRII alone.

Rats were examined at 6 h, and macroscopic skin lesions were analyzed (blind by independent observers) using two criteria: (*a*) size of the lesion; the area in mm^2 was calculated by multiplying the transverse width in two perpendicular directions; and (*b*) "total score" reflecting the severity of each lesion; a minor score of 0 (nil), 1 (mild), 2 (moderate), or 3 (severe) was given for two separate parameters (oedema and erythema), and a total score for the lesion was assigned by adding the two minor scores. Skin biopsies at injected sites, taken through the center of the lesion, were fixed in 10% buffered formalin, stained with hematoxylin-eosin (Department of Pathology, Austin Hospital) and examined for edema, polymorphonuclear, and mononuclear cell infiltrate.

Statistical Analysis. A statistical comparison of the size and scores of the rsFcγRII-treated skin and the controls (PBS and KLH treatment) was performed by a one-way analysis of variance (67); $p < 0.05$ was considered significant.

Results

Production and Purification of rsFcγRII. The rsFcγRII cDNA construct was transfected into CHO cells and resulted in a soluble recombinant protein that was secreted into the cell culture supernatant. Maximally expressing amplified transfected CHO cells (Clone 1.5) were grown in 100 μM MSX and produced 4–8 μg of purified rsFcγRII per ml of cell culture supernatant. Immunoaffinity purification using human IgG Fc binding utilized the natural physiological ligand for the receptor and gentle elution conditions enabled maximal yield, with minimal loss of Fc binding capacity of the recombinant protein. An eluted sample analyzed by SDS-PAGE is shown (Fig. 1) and appears as a ~31,000 M_r band consis-

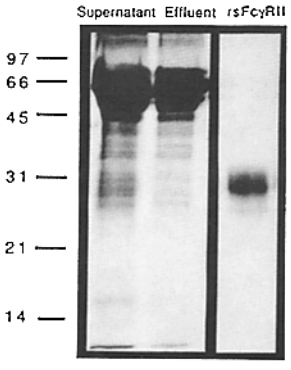

Figure 1. Samples from a typical purification profile of the rsFcγRII subjected to 13% SDS-PAGE under reducing conditions. The starting cell culture (lane *1*), the effluent from the column (lane *2*), and the eluted rsFcγRII (lane *3*) are shown. Molecular weight markers (kD) are indicated on the left.

tent with the predicted 20,000 M_r of the rsFcγRII polypeptide chain and the extra size from two N-linked carbohydrate structures. The rsFcγRII was >95% pure as analyzed by SDS-PAGE. The stability of the rsFcγRII in tissue culture supernatant was measured by the ELISA for FcγRII, and showed it was stable (mAb epitopes intact) at 4, −20, and −70°C for 6 mo. Functional activity of the rsFcγRII, demonstrated by inhibition of EA rosette formation, was completely stable for at least 1 mo at 4°C (data not shown).

Structure of the Truncated FcγRII. RsFcγRII was deglycosylated to determine the contribution of carbohydrate to the heterogeneity. Cell culture supernatant of clone 1.5 CHO cells biosynthetically labeled with [^{35}S]methionine and cysteine was used for immunoprecipitation and the molecular weight of the deglycosylated polypeptide determined after growing the transfected CHO cells in tunicamycin. Immunoprecipitation with F(ab') or F(ab')$_2$ fragments of specific anti-human FcγRII mAbs demonstrated that the total unpurified rsFcγRII in tissue culture supernatant was heterogeneous with ~28,000–36,000 M_r (Fig. 2), whereas the IgG-purified rsFcγRII (Fig. 1) was more homogeneous. However, after tunicamycin treatment, a homogeneous ~23,000 M_r band under reducing conditions (Fig. 2) and ~21,000 M_r in nonreducing conditions (data not shown) was detected, which agrees with the predicted molecular mass of 20,000 and demonstrated that the heterogeneity of the total rsFcγRII protein was due to heterogeneity within the N-linked carbohydrate, rather than proteolytic degradation or partial translation of the mRNA. The five mAbs used (IV.3, CIKM5, 8.2, 8.7, and 8.26) (Fig. 2) previously have been shown to divide into four clusters, each cluster defining a separate epitope on the extracellular domains of FcγRII (53). Cluster 1 (8.2 and CIKM5) defines an epitope with determinants in

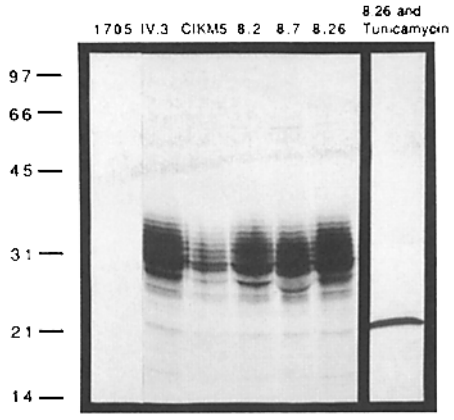

Figure 2. Immunoprecipitation of the glycosylated and deglycosylated rsFcγRII analyzed on 13% SDS-PAGE gel under reducing conditions, with molecular weight markers (kD) on the left. Supernatant from [^{35}S]methionine/cysteine-labeled transfected CHO cells were immunoprecipitated using: 1705 F(ab')$_2$ control mAb (lane *1*), IV.3 F(ab') mAb (lane *2*), CIKM5 F(ab')$_2$ mAb (lane *3*), 8.2 F(ab')$_2$ mAb (lane *4*), 8.7 F(ab')$_2$ mAb (lane *5*), and 8.26 F(ab') mAb (lane *6*). Immunoprecipitation from supernatant using tumicamycin-treated secreting CHO cells, with mAb 8.26 F(ab') is shown in lane 7.

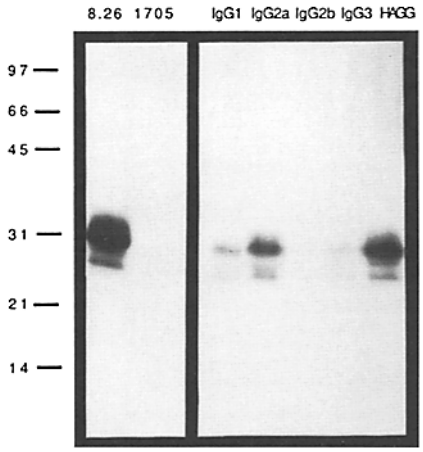

Figure 3. 13% SDS-PAGE analysis of immunoprecipitated ^{125}I-labeled rsFcγRII under reducing conditions using: mAb 8.26 F(ab') (lane *1*); mAb 1705 F(ab')$_2$ (lane *2*); whole murine, IgG1 mAb l-1 (lane *3*); IgG2a mAb 1302 (lane *4*); IgG2b mAb 1480 (lane *5*); IgG3 mAb 1308 (lane *6*); and HAGG (lane 7). Molecular weight markers (kD) are indicated on the left.

both domains 1 and 2 of FcγRII and lies distant from the Fc binding region, and clusters 2 (8.26), 3 (IV.3), and 4 (8.7) detect three additional epitopes contained in the second extracellular domain only. All five mAbs recognize the heterogenous glycosylated 31-kD rsFcγRII protein. Detection of the rsFcγRII by the mAbs, particularly mAbs from cluster 1 which define a combinatorial epitope, indicates that the

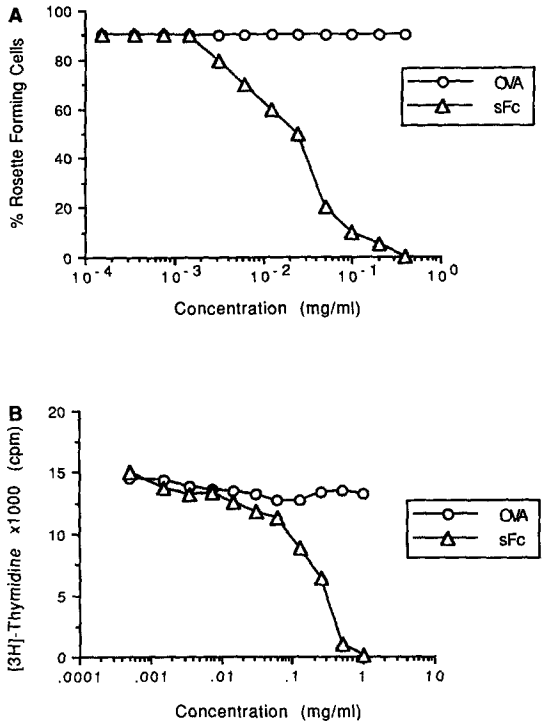

Figure 4. (*A*) Inhibition of EA rosette formation by rsFcγRII (*sFc*) using K562 FcγRII$^+$ target cells; and (*B*) inhibition of the anti-Leu4–induced T cell proliferation assay using rsFcγRII (*sFc*) or control protein (*OVA*).

tertiary structure of rsFcγRII resembles that of the membrane-bound FcγRII. Since the ligand-purified rsFcγRII was homogeneous and functional, in that it was purified by IgG aggregates (Fig. 1), only this material was used for further studies.

IgG Fc Binding by rsFcγRII. The Fc binding capacity of the purified rsFcγRII was examined by ^{125}I radiolabeling the rsFcγRII and immunopurification with HAGG or murine IgG1, IgG2a, IgG2b, and IgG3 mAbs; mAbs 8.26 F(ab') (positive control) and $F(ab')_2$ fragments of 1705 (Fc binding specificity control) were included (Fig. 3). SDS-PAGE analysis revealed the same 31-kD protein with the anti-FcγRII mAb 8.26, murine IgG1, IgG2a, and HAGG. Binding to murine IgG2b was variable as no binding was detected using the IgG2b mAb, 1480 (Fig. 3), although another murine IgG2b mAb exhibited some binding (data not shown). There was very little binding to IgG3 and no binding to the $F(ab')_2$ fragments of the nonreactive control mAb 1705 indicating that the binding of the purified rsFcγRII to IgG was specifically through the interaction of the Fc portion of IgG with the soluble receptor.

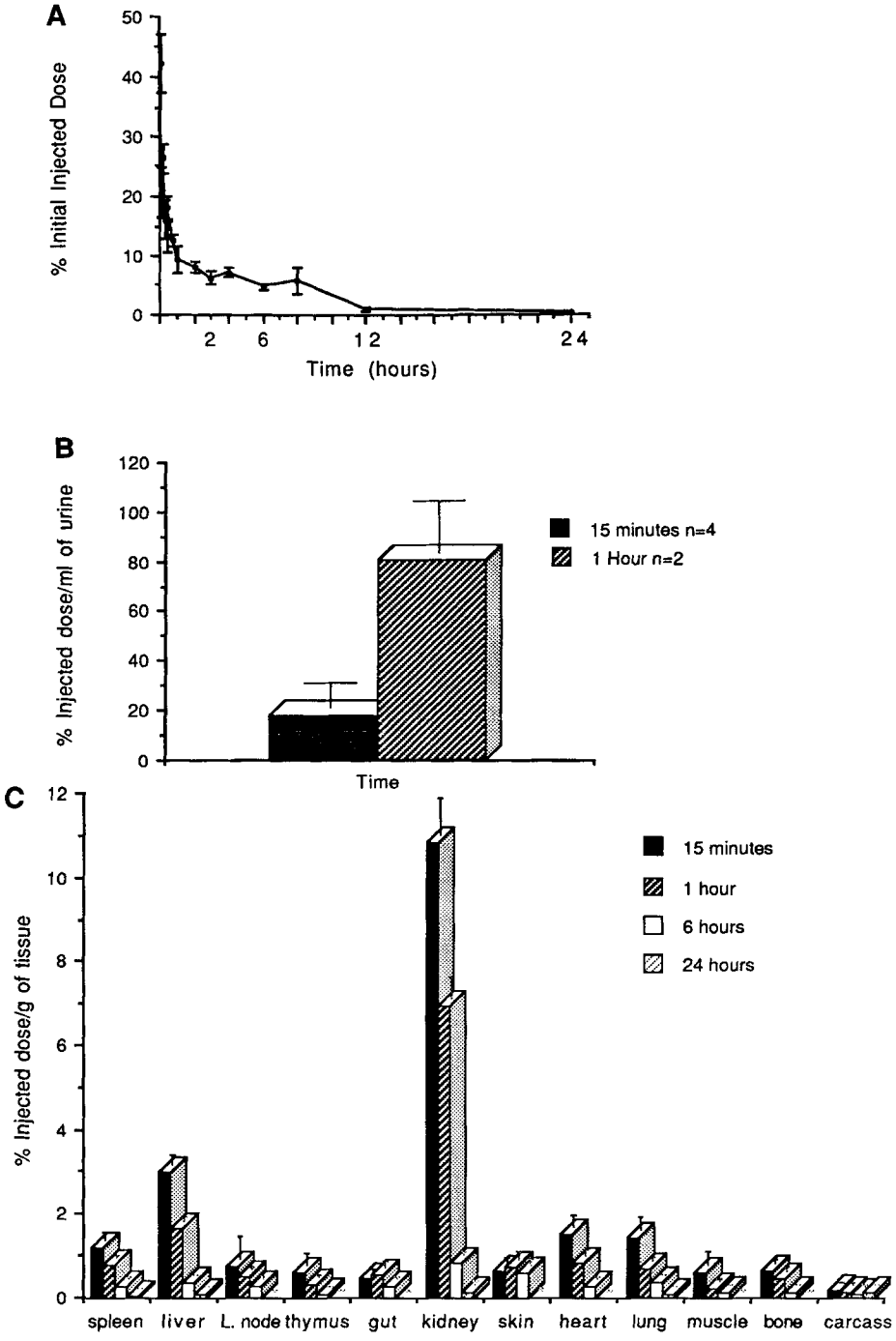

Figure 5. Blood clearance and biodistribution of ^{125}I-rsFcγRII. (*A*) Blood clearance of ^{125}I-rsFcγRII; percent initial injected dose is shown on the y-axis and time (*hours*) on the x-axis. (*B*) Urine ^{125}I-rsFcγRII levels, expressed as percent initial injected dose/ml of urine, at 15 min (n = 4) and 1 h (n = 2). (*C*) Biodistribution in various organs showing the percent initial injected dose/gram of tissue on the y-axis. Error bars, SE.

The murine IgG binding specificity of the rsFcγRII differs from the membrane-bound human FcγRII; rsFcγRII binds murine IgG2a>IgG1>>IgG2b, and IgG3. It should be noted that the HFc3.0 cDNA encodes a histidine at position 131 and poor immunoprecipitation with the murine IgG1 mAb seen in Fig. 3 would be expected as this is a "low responder" FcγRII (68–70).

The binding of rsFcγRII to immune complexes was demonstrated by its ability to inhibit the binding of EAs to cell surface FcγRII. Incubation of EAs with rsFcγRII completely inhibited the formation of EA rosettes with K562 cells, with a 50% inhibitory final concentration of 20 μg/ml of purified rsFcγRII (Fig. 4 *A*). In addition, rsFcγRII inhibited the anti-Leu4–induced T cell proliferation assay by 50% at a final concentration of 125 μg/ml, indicating the soluble receptor has the capacity to block the binding of immune complexes to FcγRII on peripheral blood monocytes (Fig. 4 *B*). We also tested the ability of rsFcγRII to inhibit an antibody-dependent, cell-mediated cytotoxicity assay and a C'-mediated cell lysis assay using rabbit C' and a murine mAb. No inhibition was observed in either assay (data not shown).

In summary, the physical and functional characteristics of the rsFcγRII are similar to membrane-bound FcγRII: (*a*) rsFcγRII is structurally related to membrane-bound FcγRII as indicated by intact epitopes for the binding of mAbs to membrane-bound FcγRII; and (*b*) rsFcγRII binds IgG through its Fc region, although the murine isotope binding specificity has been altered compared to membrane-bound human FcγRII. It is noteworthy that the general characteristics of human FcγRII in relation to binding specificities of IgG have been derived from studies using few cell types and may not apply in all cases (2).

Blood Clearance and Biodistribution of rsFcγRII. The in vivo characteristics of rsFcγRII were studied in mice by analyzing the blood clearance and biodistribution of intravenously administered ^{125}I-rsFcγRII. The blood clearance studies demonstrate that ^{125}I-rsFcγRII is rapidly eliminated from the circulation in a biphasic manner with an α-phase *t*1/2 of 25 min and a β-phase *t*1/2 of 4.6 h; after 24 h the ^{125}I-rsFcγRII was totally eliminated (Fig. 5 *A*). Groups of four mice were also injected intravenously and various organs were counted for radioactivity (Fig. 5 *C*). There was a rapid early concentration of ^{125}I-rsFcγRII in the kidney at 15 min and 1 h, with a subsequent fall at 6 and 24 h. No other organ exhibited tissue-specific accumulation. The rapid clearance from the circulation, associated with high concentrations in the kidney, suggested the recombinant protein was excreted predominantly through the kidney. Studies of urine demonstrate high levels of I^{125}-rsFcγRII at 1 h, indicating this to be the major route of excretion (Fig. 5 *B*). The effect of route of administration was studied by giving either an i.p. or i.v. dose of ^{125}I-rsFcγRII. An identical pattern of rapid blood clearance was seen with both i.p. and i.v. injections (data not shown).

Inhibition of the Arthus Reaction. Rats given i.v. OVA (5 mg) followed by intradermal rabbit anti-OVA IgG (500 μg) mixed in PBS developed a visible Arthus reaction within 2 h of the intradermal injection and at 6 h were characterized macroscopically by an erythematous and edematous inflamed area of skin measuring 30 mm^2 determined from the width of the lesion in two perpendicular transverse directions (Fig. 6). Histologically, the tissue section from the site of the lesion showed an inflammatory infiltrate of polymorphonuclear cells, and to a lesser extent mononuclear cells, particularly around venules in the dermis (Fig. 7). Specificity of the RPAR was demonstrated by giving rats i.v. OVA, intradermal rabbit IgG, or rsFcγRII, all given separately to individual rats, or nonimmune rabbit IgG intradermally together with i.v. OVA. In all cases, the RPAR did not develop (data not shown).

To test the effect of rsFcγRII, OVA-injected rats were then given a constant amount of rabbit anti-OVA IgG (500 μg) mixed with rsFcγRII at varying doses (50–500 μg) in a final volume of 100 μl intradermally. When rsFcγRII was given with the rabbit anti-OVA IgG, a specific and significant dose-dependent inhibition of the size (using 500 μg of rsFcγRII, $p < 0.05$) and the score (using 500 and 150 μg of rsFcγRII,

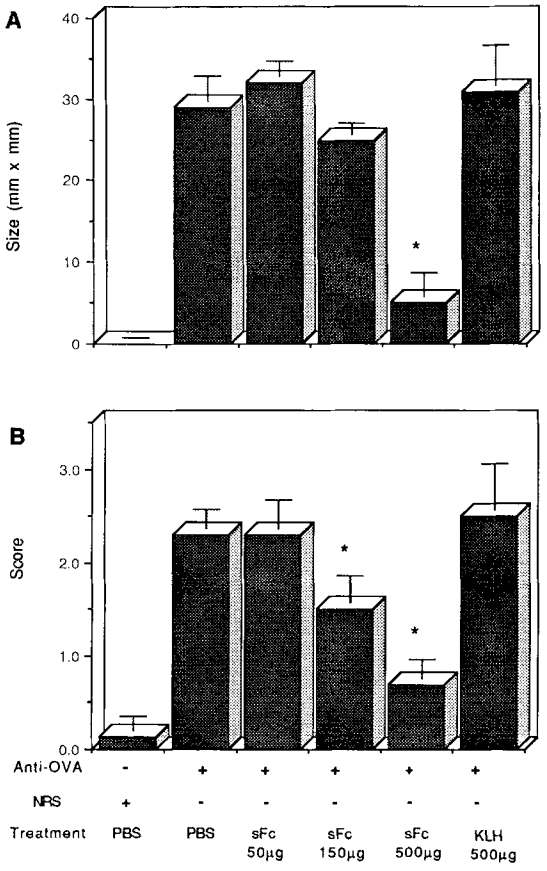

Figure 6. Size (*A*) and score (*B*) of the Arthus lesions are shown on the y-axis, using (x-axis): 500 μg of normal rabbit serum (*NRS*) (n = 7); 500 μg of anti-OVA with PBS (n = 27); 500 μg of anti-OVA with 50 μg of rsFcγRII (*sFc*) (n = 6); 500 μg of anti-OVA with 150 μg of sFc (n = 8); 500 μg of anti-OVA with 500 μg of sFc (n = 14); and 500 μg of anti-OVA with 500 μg of KLH (n = 4). (n) Number of injection sites using multiple rats. Error bars represent SE; (*) $p < 0.05$ compared to the PBS- and KLH-treated skin.

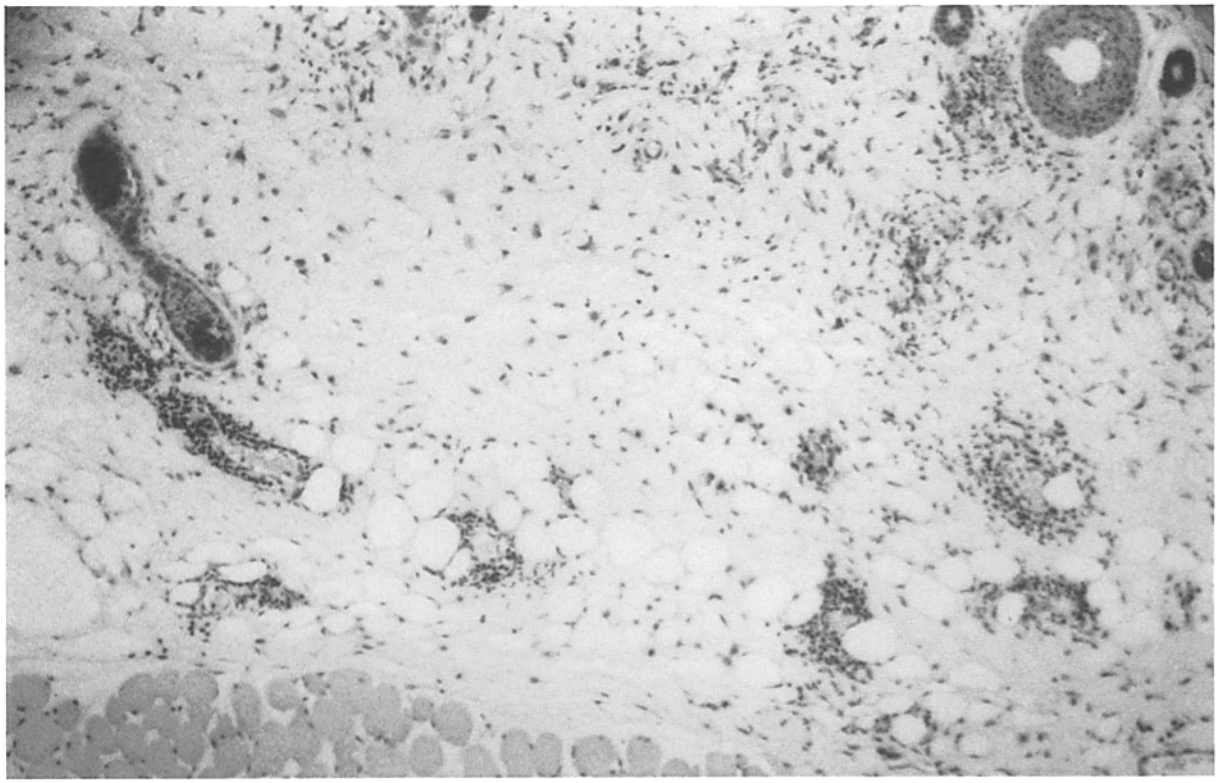

Figure 7. Histology of the Arthus reaction in skin, treated with PBS (positive control) 6 h after injection, showing intense perivascular neutrophil infiltrate. ×100.

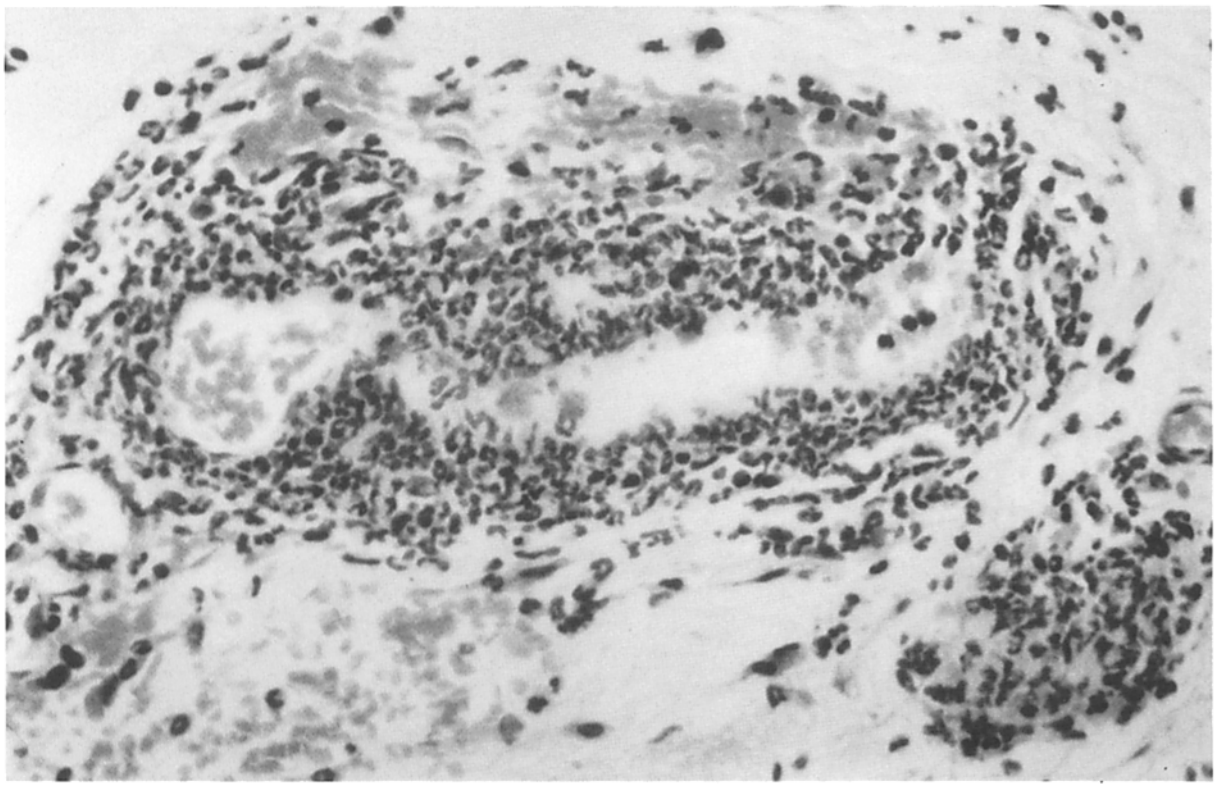

Figure 8. Histology of the PBS-treated skin demonstrating a typical perivascular neutrophil infiltrate with extravasation of erythrocytes. ×400.

both $p < 0.05$) of the Arthus lesion was observed, compared to PBS- and KLH (irrelevant protein control) -treated lesions (Fig. 6). The results represent multiple injections using a number of different rats. Histological sections taken from the sites treated with the rsFcγRII mixed with the rabbit anti-OVA showed a marked reduction in neutrophil accumulation, margination, infiltration around venules, and erythrocyte extravasation compared to PBS-treated skin (Figs. 7–9). The mild, persistent neutrophil infiltrate in the rsFcγRII-treated skin is likely to be a consequence of the short *t1/2* of rsFcγRII and C activation.

Discussion

A functional rsFcγRII protein was made by genetically deleting the transmembrane and cytoplasmic domains of a human FcγRII protein, and using a novel eukaryotic expression system in CHO cells, resulted in the secretion of the rsFcγRII into the tissue culture supernatant, enabling subsequent purification. The recombinant glycoprotein was shown to have in vitro and in vivo activity. The truncated 31-kD recombinant protein containing the two extracellular domains of FcγRIIa could bind human, mouse, and rabbit Ig by their Fc portions, and completely inhibited the binding of immune complexes to cell surface FcγRII. The interaction of immune complexes with cell surface FcγRII initiates a number of Fc-mediated effector mechanisms including inflammatory responses, and therefore, a classical model of immune complex disease (the Arthus reaction) was used to investigate the possible in vivo therapeutic properties of the human rsFcγRII. The RPAR was significantly inhibited by rsFcγRII in a dose-dependent manner, with less extensive macroscopic lesions and histological inflammatory cell infiltrates demonstrated in treated areas.

The first part of the study addressed the problem of producing large quantities of purified functional rsFcγRII. The mammalian expression vector, pEE6/CMV/GS, was utilized as it gives high levels of expression (51). In this study, all the original clones did not survive in >15 μM MSX, although amplification of the initial clones using 100–1,000 μM MSX produced survivors in 100 μM MSX, and a four-fold increase in rsFcγRII production was noted in the amplified clones (4–8 mg/liter of supernatant). This could be increased by further selection and attention to the culture medium, however the production level sufficed for our studies. Kinetic studies (data not shown) using supernatant and cell lysates from biosynthetically labeled transfected CHO cells was performed to exclude the possibility of a defective secretory mechanism resulting in intracellular accumulation of rsFcγRII. These studies demonstrated that the synthesized rsFcγRII first appeared in the tissue culture supernatant within 1 h of the initial [^{35}S]methionine/cysteine pulse and was totally secreted in 2 h, with no intracellular accumulation.

RsFcγRII is a functional receptor and clearly binds IgG

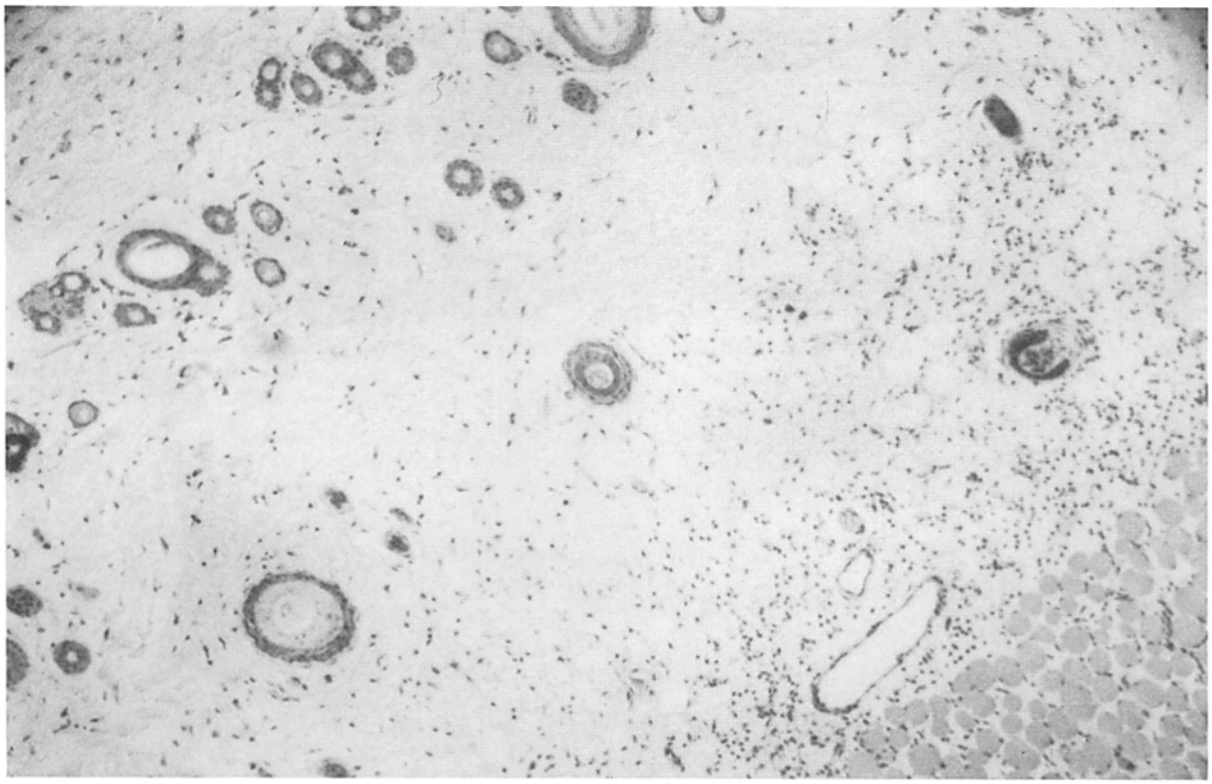

Figure 9. Histology of rat skin treated with 500 μg of rsFcγRII showing reduction in the inflammatory reaction. ×100.

through the Fc portion, albeit with altered properties compared to the native cell surface FcγRII. Membrane-bound human FcγRIIa is a low affinity receptor (K_a = 10^6 M^{-1}) for monomeric IgG, and the low responder form binds murine IgG2b>>IgG2a,IgG3 compared to rsFcγRII which binds murine IgG2a>IgG1>>IgG2b, or IgG3 (Fig. 3). The poor binding of rsFcγRII to murine IgG1 is consistent with the "low responder" phenotype which is a genetically determined polymorphism resulting in the expression of two allelic variants which differ in their ability to bind murine IgG1; high responders bind murine IgG1 more avidly than low responders (62, 63). Low responder FcγRII, like HFc3.0 used herein, contains histidine at position 131 whereas high responder FcγRII contains arginine in this position (68–70). Furthermore, some changes in fine specificity may not be unexpected since significant changes in carbohydrate side chains from the native receptor could alter the conformation and therefore the binding properties, as reported for other receptors (71). This may account for the difference in IgG2a and IgG2b binding reported here and in the literature. The absence of the transmembrane and cytoplasmic tail may also influence structural and functional aspects of the soluble receptor, however, the essential properties have been maintained since the rsFcγRII binds IgG via the Fc portion and is recognized by mAbs which detect conformational changes in cell surface FcγRII.

RsFcγRII is a relatively small polypeptide (31,000 M_r) with ~25% of its molecular mass attributable to N-linked glycosylation. Small molecular weight compounds would be expected to be easily filtered through the glomerulus and indeed, the rsFcγRII is rapidly cleared from the circulation through the kidneys (Fig. 5). One might expect a longer in vivo half-life if rsFcγRII bound to IgG in serum, however several factors may influence this interaction: (*a*) rapid renal clearance not allowing the rsFcγRII–IgG interaction to approach equilibrium in serum; and (*b*) affinity of monomeric IgG for rsFcγRII. The short half-life of the rsFcγRII in vivo will clearly limit the use of the protein as a parenterally administered therapeutic agent, but in its present form, could be useful for local therapy such as in inflamed joints in rheumatoid arthritis. The RPAR model used in this study produced a localized area of dermal inflammation and demonstrates that rsFcγRII administered concomitantly with anti-OVA antibody significantly reduces the immune complex–mediated tissue damage. Many cytokines with rapid clearance from the circulation, resulting in short-lived pharmacological effects, have been successfully modified to increase their half-life and subsequent potency of the compound in vivo (72–73). A similar approach with the rsFcγRII protein (currently in progress) would broaden its therapeutic application to a parenterally administered reagent.

In two other reports (66, 74) C′ regulating proteins (soluble C receptor type I and decay accelerating factor) did not completely inhibit the Arthus reaction. In one study, the RPAR model used was similar to that used here (66). The persisting inflammatory response seen after treatment with the C′ regulators is likely to be due to "unblocked" FcγR-mediated inflammatory response. Similarly, in the study described herein, inhibition of the inflammatory response was profound but incomplete. This probably relates to the short *t1/2* and/or inability to inhibit C activation. Since both FcγR- and C′-mediated inflammatory responses are critical in the pathogenesis of immune complex–induced tissue injury, complete inhibition of the inflammatory response presumably requires inhibition of both pathways. Studies are currently in progress to determine the potency of "combined" therapy.

The mode of action of rsFcγRII is not clear but could be mediating the anti-inflammatory response by several different mechanisms. One likely site of inhibition is the activation of neutrophils and monocytes with subsequent release of inflammatory mediators which is known to be signaled through the interaction of immune complexes with cell surface FcγRII, and we have shown in this study that rsFcγRII completely inhibits the binding of EA immune complexes to cell surface FcγRII and furthermore inhibits the anti-Leu4–induced T cell proliferation assay which results from the binding of rsFcγRII to the Fc portion of anti-Leu4 bound to T cells (Fig. 4). A second possible mechanism is the interruption of the C′ cascade known to release many inflammatory proteins (e.g., C5a) which induce chemotaxis and neutrophil activation. Murine soluble FcγRII has been shown to inhibit C′-mediated lysis of SRBC by IgG in vitro (24). We found that rsFcγRII did not inhibit C′-mediated cell lysis or antibody-dependent cell-mediated killing by mononuclear cells. Antibody-dependent cell-mediated lysis is a function of FcγRIII, and therefore inhibition by rsFcγRII was not expected. It is likely that complement is activated, which leads to neutrophil chemotaxis, but neutrophil activation via FcγRII by immune complexes, which results in inflammatory mediator release, is inhibited. Platelets have been shown to initiate or augment immunologically mediated inflammation and tissue injury by the activation of circulating platelets through the interaction of immune complexes with cell surface FcγRII (8). Inhibition of platelet activation and aggregation could also reduce the inflammatory response, and we are currently investigating the role of rsFcγRII in modifying platelet function.

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