IgM Anti-$Fc_\gamma R$ Autoantibodies Trigger Neutrophil Degranulation

By Peter Boros,* Joseph A. Odin,* Tai Muryoi,‡ Sandra K. Masur,§ Constantin Bona,‡ and Jay C. Unkeless*

*From the Departments of *Biochemistry, ‡Microbiology, and §Physiology and Biophysics, The Mount Sinai School of Medicine, New York, New York 10029*

Summary

Anti-$Fc_\gamma R$ IgM monoclonal antibodies (mAbs) isolated from lipopolysaccharide-stimulated spleen cells from tightskin (TSK) mice were found to be polyspecific, reacting with a wide variety of molecules, including double-stranded DNA, topoisomerase, RNA polymerase, and different collagen types. Approximately 60% of the polyspecific IgM mAbs have anti-$Fc_\gamma R$ specificity. These anti-$Fc_\gamma R$ mAbs induce the release of hydrolases from both azurophil and specific granules of human neutrophils. 25–45% of the total cellular content (determined in Nonidet P-40 lysates) of neutrophil elastase, 10–25% of β-glucuronidase, and 30–50% of alkaline phosphatase was released after incubation with the mAbs. The degranulation process was accompanied by dramatic morphological changes shown by scanning and transmission electron microscopy. The release of hydrolytic enzymes stimulated by the IgM anti-$Fc_\gamma R$ mAbs was inhibited by preincubation of neutrophils with Fab fragments of either anti-human $Fc_\gamma RII$ (IV.3) or anti-human $Fc_\gamma RIII$ (3G8) mAbs. The binding of the anti-$Fc_\gamma R$ TSK mAbs to human neutrophils was inhibited by Fab fragments of mAb 3G8. However, we found that the TSK anti-$Fc_\gamma R$ mAbs do not bind to human $Fc_\gamma RII$ expressed in either CHO cells or the P388D1 mouse macrophage cell line. Since the enzyme release could be inhibited by Fab fragments of mAb IV.3, we suggest that the signal transduction may require $Fc_\gamma RII$ activation subsequent to crosslinking of the glycan phosphatidyl inositol–anchored $Fc_\gamma RIII$-1. These data demonstrate for the first time that polyspecific autoantibodies with $Fc_\gamma R$ specificity can trigger neutrophil enzyme release via human $Fc_\gamma RIII$-1 in vitro and indicate a possible role for such autoantibodies in autoimmune inflammatory processes.

Organ injury due to inflammatory processes is a fundamental sign of many autoimmune diseases. Autoantibodies and immune complexes that are the hallmark of autoimmunity can trigger inflammation by several pathways. Immune complexes can activate the complement system resulting in generation of chemotactic peptides and deposition of C3b and C3bi, which are ligands for complement receptors on neutrophils and macrophages. Immune complexes may also interact directly with $Fc_\gamma Rs$ on effector cells, resulting in release of hydrolases, activated oxygen intermediates, and cytokines (1, 2). Other autoantibodies upon interaction with their antigens such as acetylcholine receptor directly trigger pathogenic events (3).

We have recently reported the presence of high levels of anti-$Fc_\gamma R$ autoantibodies in different mouse strains prone to autoimmune diseases such as NZB, NZB/NZW, Tightskin (TSK)[1], and viable-motheaten (4). Moreover, some IgM mAbs generated from spleen cells of these animals bound to murine $Fc_\gamma RII$. The mAbs with $Fc_\gamma R$ specificity comprise a subset (60%) of polyspecific IgM mAbs. Both serum from autoimmune mice and IgM anti-$Fc_\gamma R$ mAbs were able to inhibit specifically the binding of immune complexes in vitro to macrophages. These anti-$Fc_\gamma R$ autoantibodies may be responsible for the paralysis of macrophage $Fc_\gamma R$ function seen in peritoneal macrophages isolated from autoimmune mice (5).

Human anti-$Fc_\gamma R$ receptor antibodies have been demonstrated in SLE and juvenile neutropenia (6–8). Furthermore, in diseases such as Sjogren's syndrome, rheumatoid arthritis, and lupus (9), there is often a paralysis of $Fc_\gamma R$ function similar to that observed in the murine models for autoimmunity. Functional studies are required to assess the importance of these polyspecific anti-$Fc_\gamma R$ antibodies in the pathology of autoimmune disease. In this report, we present evidence that anti-$Fc_\gamma R$ autoantibodies have a profound effect on human neutrophils, resulting in release of hydrolytic enzymes contained in both specific and azurophil granules.

[1] *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; GPI, glycan phosphatidyl inositol; PSS, progressive systemic sclerosis; TSK, tightskin.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/06/1473/10 $2.00
Volume 173 June 1991 1473–1482

Materials and Methods

Monoclonal Antibodies from TSK Mice. Splenic lymphocytes from 2- and 10-mo-old TSK mice, stimulated with LPS (25 μg/ml) for 2 d, were fused with SP2/0 myeloma cells as described (10, 11). Supernatants from hybridomas were tested by an ELISA for binding to truncated mouse $Fc_\gamma RII\beta$ (12) that was coated onto microtiter wells (4). The truncated $Fc_\gamma RII\beta$ contained only the extracellular domains of the receptor. To prevent nonspecific binding via the Fc domain, the truncated $Fc_\gamma R$ was denatured by reduction and alkylation. 5 of 440 hybridomas had specificity for $Fc_\gamma R$, and these hybridomas were expanded and cloned by limiting dilution. Antibodies were purified by affinity chromatography on a rat anti-mouse κ-specific mAb Sepharose-4B column. All the anti-$Fc_\gamma R$ mAbs were of the IgM class.

Isolation of Human Neutrophils. Peripheral blood drawn from healthy individuals was anticoagulated with heparin and diluted 1:3 with HBSS. The blood (20 ml) was carefully layered on top of a two-step gradient made from 1.119 g/ml Ficoll and 1.077 g/ml Ficoll (10 ml each) (Sigma Chemical Co., St. Louis, MO) (13). After centrifugation (23 min, 24°C, 1,000 *g*) in a swinging bucket rotor, the neutrophils were collected, washed, and resuspended in RPMI containing 10% FCS.

Expression of Human $Fc_\gamma RII$ in P388D1 and CHO Cells. We obtained a human (hu) $Fc_\gamma RIIa$ cDNA (14, 15) that was cloned into the EcoRI restriction site of the pGEM-4 vector from Dr. Kochan (Hoffman LaRoche, Nutley, NJ). The cDNA was then subcloned into the EcoRI site of pcEXV-3 (16), a eukaryotic expression vector with an SV40 early gene promoter. Plasmid DNA from both positive (expressing) orientation and negative orientation $huFc_\gamma RIIa$-containing bacterial transformants was purified on a CsCl gradient (17) for use in calcium phosphate–DNA coprecipitate transfections as described (18). Dihydrofolate reductase (DHFR)-negative CHO cells (DG44) were cotransfected with huFcRIIa-pcEXV-3 and a DHFR minigene–containing plasmid, pMG1 (19). CHO transfectants were selected using hypoxanthine-deficient media. P388D1 cells were cotransfected with huFcRIIa-pcEXV-3 and LK444 (20), a plasmid with a neomycin resistance gene. The only modification of the calcium phosphate coprecipitation method required to transfect the P388D1 cells was the addition of 100 μM chloroquine (Sigma Chemical Co.) to the transfection media. P388D1 transfectants were selected by adding 200 mg/liter of Geneticin (Sigma Chemical Co.) to the growth media. The ability of transfectants to bind mAb IV.3, an anti-huFcRII antibody, was analyzed on an Epics cytofluorograph (Coulter Electronics Inc., Hialeah, FL) with three decades of amplification (21).

Binding Studies. mAbs 3G8, TSK1, TSK22, TSK26, and TSK40 were conjugated with FITC (22). 5×10^5 neutrophils were incubated in 0.25 ml with the antibodies (1 μg/ml) for 45 min on ice. The cells were then washed and fixed with 3% paraformaldehyde in PBS. Analysis was done on an Epics cytofluorograph with three decades of amplification. Mean fluorescence peak channels were converted to relative fluorescence to facilitate comparison of inhibition and binding studies. To quantify the amount of FITC-Ig bound per cell, the fluorescence of appropriate dilutions of the FITC-conjugated Ig and stained cells was determined in a fluorescence spectrometer (650-10S; The Perkin-Elmer Corp., Norwalk, CT) with excitation and emission monochromators set at 480 and 520 nm, respectively.

Stimulation of Neutrophils by Anti-$Fc_\gamma R$ Autoantibodies. Neutrophils suspended in RPMI supplemented with 10% FCS were plated in 24-well tissue culture plates (Costar Electronics Inc.) at 10^6 cells/well. The original medium was removed after 30 min and replaced with 1 ml of Ig (0.01–10 mg/ml) diluted in RPMI containing 10% FCS. Aliquots of cell supernatants were taken at intervals to follow the course of enzyme release. In blocking experiments to study the effect of the anti-$Fc_\gamma RIII$ mAb 3G8 and the anti-$Fc_\gamma RII$ mAb IV.3, the mAbs (or their Fab fragments) were added to the cells for 1 h before they were replaced with the anti-$Fc_\gamma R$ autoantibodies. mAb IV.3 and its Fab fragment were purchased from Medarex (Hanover, NH). mAb 3G8 and 3G8-Fab were prepared in our laboratory as described (23).

Enzyme Assays. All enzyme determinations were carried out on 96-well microtiter plates in triplicate. To quantify enzyme release, dilutions of neutrophil-conditioned supernatants or lysates were mixed with appropriate substrates and buffers and read in a microplate reader (MR600; Dynatech Laboratories Inc., Alexandria, VA). β-glucuronidase was assayed by cleavage of phenolphthalein-β-glucosiduronic acid (Sigma Chemical Co.) as described (24). Supernatant (50 μl) was added to the substrate (30 μl of 3.3 mM phenolphthalein-β-glucosiduronic acid in 0.067 M acetate buffer, pH 4.5). After incubation (3 h, room temperature) the reaction was developed by addition of glycine buffer (20 μl, 0.4 M, pH 10.5) and read at 540 nm. Neutrophil elastase activity was assayed by the cleavage of N-t-BOC-ala-pro-nva thiobenzyl ester (Sigma Chemical Co.) in the presence of 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) (Pierce Chemical Co., Rockford, IL), which reacts with the free sulfhydryl of the liberated thiobenzyl group (25). Supernatant (25 μl) was added to the reaction mixture (75 μl, 4.5 mM peptide, 0.33 mM Ellman's reagent, in PBS, pH 7.4, containing 10% DMSO), incubated at room temperature for 1 h, and read at 410 nm. Alkaline phosphatase activity was measured by cleavage of *p*-nitrophenyl phosphate (26). Supernatant (50 μl) was added to the reaction mixture (50 μl, 6 mM *p*-nitrophenyl phosphate, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 0.1 M glycine buffer, pH 10.0), incubated at 37°C for 1 h, and read at 410 nm. To determine total cellular enzyme content, neutrophils were lysed with 1 ml of 0.5% NP-40 for 10 min, and cleared of nuclei and particulate matter by centrifugation (20 min at 14,000 *g*). The cleared lysate supernatant was used in assays for determinations of total cellular enzyme.

Scanning Electron Microscopy. Neutrophils (10^6 cells/well) were plated on round coverslips (12 mm diameter, no. 1) (Propper SGA Scientific Inc., Bloomfield, NY) in 24-well tissue culture plates in RPMI containing 10% FCS. After 30 min, complete medium containing the different anti-$Fc_\gamma R$ antibodies was added. At intervals, the antibody-containing medium was removed and the cells were fixed for 30 min (1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) and postfixed (1% osmium in 0.1 M cacodylate buffer) (27) for 1 h. The coverslips were dehydrated, critical point dried, and viewed in a scanning electron microscope (S-530; Hitachi) at 6,000×. Some duplicate coverslips were kept in 1% cacodylate buffer after osmium treatment. These coverslips were examined using a 50× objective with Nomarsky (differential interference optics) on an Axiomat microscope (Carl Zeiss, Inc., Thornwood, NJ).

Transmission Electron Microscopy. For transmission electron microscopy, neutrophils were incubated with the anti-$Fc_\gamma R$ mAbs or controls in suspension. After fixation (2% glutaraldehyde in 0.1 M cacodylate buffer, 1 mM $CaCl_2$) for 30 min, the cells were collected by centrifugation and postfixed (1% reduced osmium in 0.1 M cacodylate buffer) (27) for 1 h on ice. The samples were dehydrated, embedded, and silver-gold sections cut on a microtome (MT-5000; Sorvall). The grids were stained with uranyl acetate and lead citrate (28, 29) and photographed at ×8,000 on an electron microscope (H-7000; Hitachi).

Results

TSK mice are considered to be a good animal model of human progressive systemic sclerosis (PSS). These mice develop a scleroderma-like syndrome, accompanied with cutaneous hyperplasia and increased transcription of collagen genes (30, 31). The old TSK mice also produce autoantibodies for topoisomerase I (11), which are characteristic for human progressive systemic sclerosis and are not found in patients with morphea or CREST syndrome (32). Because the highest levels of circulating anti-$Fc_\gamma R$ autoantibodies were found in sera of TSK mice (4), we isolated monoclonal anti-$Fc_\gamma R$ autoantibodies from TSK mice and investigated the possible functional effect of those antibodies.

Characterization of Anti-$Fc_\gamma R$ IgM Antibodies from TSK Mice. 440 LPS-stimulated hybridomas generated from old TSK mice were screened with different antigens known to be targets of scleroderma autoantibodies. All the screening for anti-$Fc_\gamma R$ mAbs was done on microtiter plates coated with reduced and alkylated truncated $Fc_\gamma RII\beta$ (12). The mAbs obtained therefore do not depend upon native conformation of the $Fc_\gamma R$ for binding, as do the two anti-$Fc_\gamma R$ mAbs 3G8 (anti-human $Fc_\gamma RIII$) and 2.4G2 (anti-murine $Fc_\gamma RII$). The mAbs that were identified in the ELISA for anti-$Fc_\gamma R$ mAbs were examined further for binding to topoisomerase I, RNA polymerase, collagen I and III, and dsDNA. The TSK antibodies we identified in the initial screen were all polyspecific, and constitute a subset (60%) of all polyspecific antibodies found, which we defined as mAbs reacting with three disparate antigens. Within the subset of anti-$Fc_\gamma R$ polyspecific mAbs, there were differences in the fine specificity. TSK1, TSK22, and TSK23, for example, were not reactive with dsDNA (results not shown). We were concerned, in particular, with the possible rheumatoid factor activity of the mAbs, which would complicate analysis of the effect the mAbs had on neutrophil function. The anti-$Fc_\gamma R$ mAbs did not exhibit binding to murine IgG1.

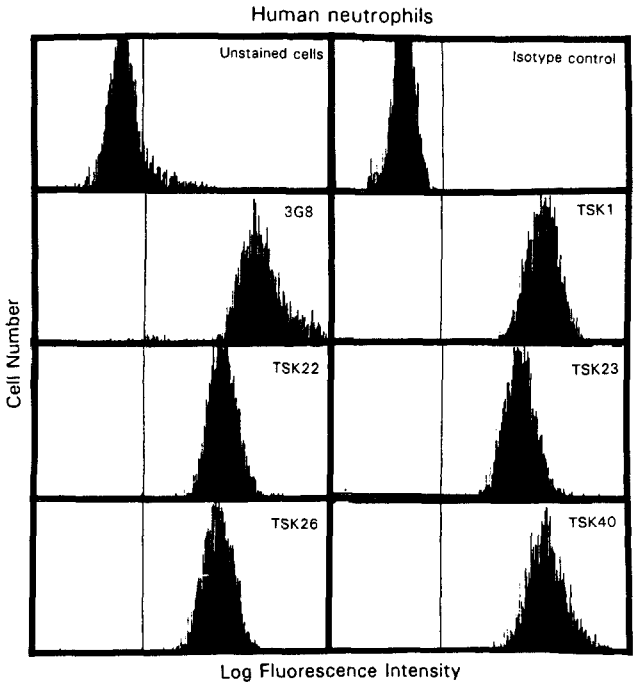

Figure 1. Staining of human neutrophils with IgM anti-$Fc_\gamma R$ mAbs. Human neutrophils (5×10^5 cells/sample) were incubated with directly FITC-labeled mAbs 3G8, TSK1, TSK22, TSK23, TSK26, and TSK40 (1 μg/ml) for 45 min on ice. The mean fluorescence intensities of the different samples were: unstained, 2.428; isotype control (mouse IgM), 8.867; 3G8, 208.4; TSK1, 159.1; TSK22, 85.54; TSK23, 85.54; TSK26, 76.79; TSK40, 142.8.

Table 1. *Binding of FITC-TSK1 to Neutrophils and Inhibition by mAb 3G8 Fab*

mAb	Fluorescence intensity
Unstained cells	6.7
Mouse IgM-FITC	14.6
3G8-FITC	
5 μg/ml	143.2
0.5 μg/ml	90.2
TSK1-FITC	
5 μg/ml	144.7
0.5 μg/ml	73.8
+3G8 Fab 0.2 μg/ml	61.4
1 μg/ml	46.1
3 μg/ml	31.4

Neutrophils (10^6 cells/sample) were incubated with FITC-conjugated antibodies, as described, on ice for 45 min. For the inhibition experiment, cells were preincubated with indicated concentrations of 3G8 Fab for 1 h, washed twice by centrifugation, and stained with FITC-TSK1. Arbitrary fluorescence units from the fluorescence spectrometer are shown.

All the mAbs specific for mouse $Fc_\gamma R$ bind strongly to human neutrophils (Fig. 1). Cells stained with FITC-3G8 are ~12.5-fold brighter than the isotype control. There are differences among the staining properties of the five anti-$Fc_\gamma R$ mAbs. TSK1 and TSK40 stain somewhat more intensely than TSK22, TSK23, and TSK26. Since the mAbs were coupled directly with FITC, we could quantify the amount of antibody bound by fluorescence spectroscopy. We found that 72,000 TSK1 IgM molecules bound per neutrophil, compared with 480,000 3G8 IgG molecules per cell (Table 1). Since the IgM is decavalent, the number of IgM TSK1 molecules that bind is probably a 5–10-fold underestimate of the number of $Fc_\gamma R$ sites reactive with the antibody.

Human neutrophils express both $Fc_\gamma RII$ (CD32) and $Fc_\gamma RIII$-1, (CD16), which is a glycan phosphatidyl inositol (GPI)-anchored protein. Human neutrophils are reported to express from 150,000 (23, 33, 34) to 400,000 (35) $Fc_\gamma RIII$-1 molecules per cell. The number of $Fc_\gamma RII$ sites on neutrophils is much lower, 15,000–35,000 sites/cell (33, 34). We conclude from the number of IgM TSK1 molecules bound that the mAb must react with $Fc_\gamma RIII$. This result is con-

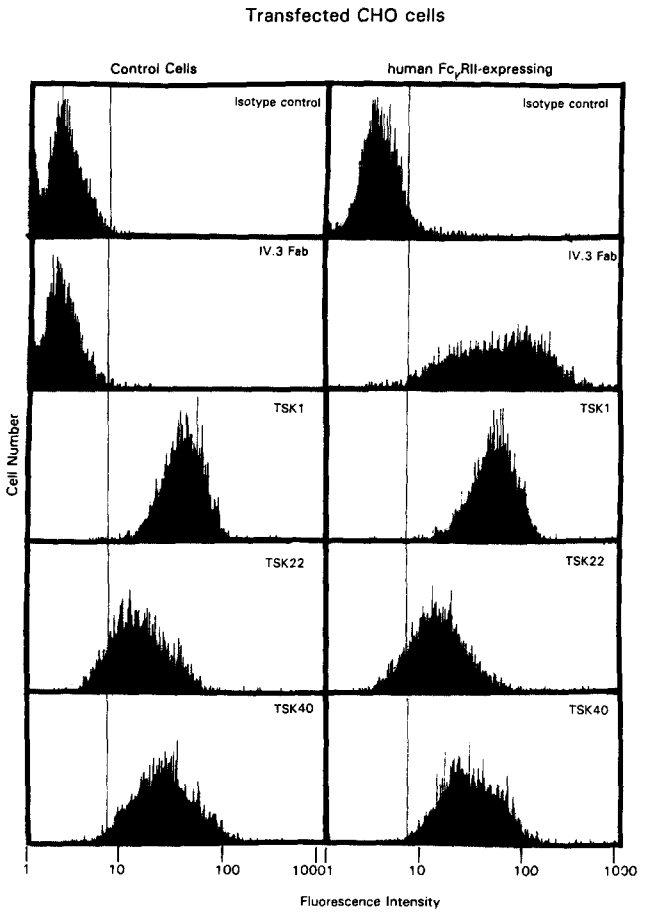

Figure 2. Binding of IgM anti-$Fc_\gamma R$ mAbs to CHO cells and CHO cells transfected with human $Fc_\gamma RII$. Cells were removed from dishes by brief treatment with trypsin, and were incubated with mouse IgM (isotope control), mAb IV.3, and different anti-$Fc_\gamma R$ IgM mAbs (1 μg/ml). Cells were then incubated with a secondary FITC anti-mouse IgM or IgG reagent. Mean fluorescence intensities of the CHO cells were: isotype control, 2.301; IV.3 Fab, 3.640; TSK1, 39.11; TSK22, 14.41; TSK40, 26.09. For the CHO cells expressing human $Fc_\gamma RII$, these values were: isotype control, 2.563; IV.3 Fab, 55.55; TSK1, 51.23; TSK22, 14.81; TSK40, 29.86.

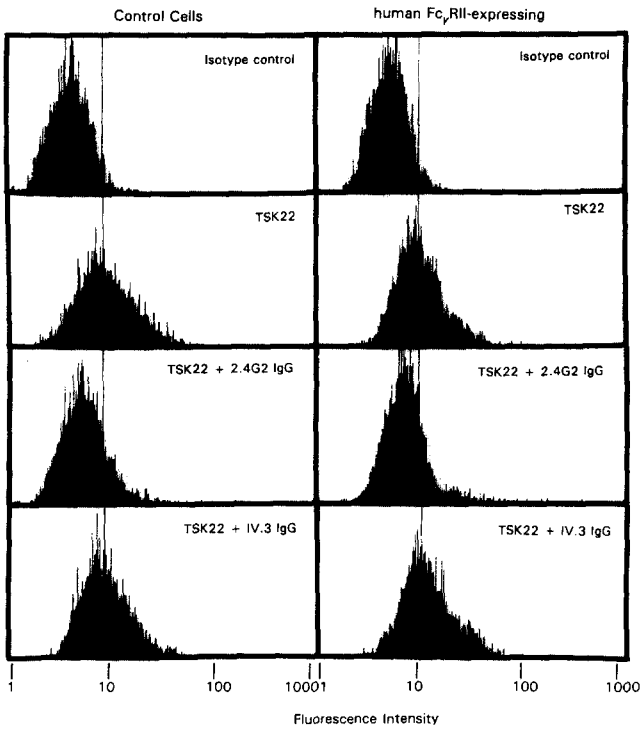

Figure 3. Binding of anti-$Fc_\gamma R$ IgM mAbs to P388D1 cells and P388D1 cells transfected with human $Fc_\gamma RII$. The PW16 clone made by transfection with human $Fc_\gamma RII$ and the original P388D1 cell line transfected with the opposite orientation of the same insert were stained with TSK22 antibody (1 μg/ml). For inhibition studies, samples from both types of cells were preincubated with either 2.4G2 or IV.3 mAbs (1 μg/ml, 45 min on ice). This step was followed by incubation with the FITC-TSK22 antibody. The values of mean fluorescence intensity for the control cells were: isotype control, 4.166; TSK22, 8.867; 2.4G2 + TSK22, 5.605; IV.3 + TSK22, 8.631. For the transformed clone: isotype control, 5.032; TSK22, 9.878; 2.4G2 + TSK22, 5.605; IV.3 + TSK22, 11.30.

firmed by the inhibition of TSK1 binding to neutrophils preincubated with increasing concentrations of 3G8 Fab (Table 1).

To determine if the various TSK antibodies also bound to human $Fc_\gamma RII$, we used transfectants expressing $Fc_\gamma RII$. We first tested a CHO line transfected with $Fc_\gamma RIIa$ (CW14) and untransfected CHO cells. The CW14 line expressed high levels of $Fc_\gamma RII$, determined by binding of mAb IV.3 (Fig. 2) compared with the untransfected control, which was the same as the isotype control. We determined by Scatchard analysis that the CW14 line expresses 2.5×10^5 $Fc_\gamma RII$ molecules/cell (data not shown). There was no difference in staining by the TSK mAbs between the control and $Fc_\gamma RII$-expressing CHO cells. The distribution of CW14 cells that stain with mAb IV.3 (specific for $Fc_\gamma RII$) is very broad, indicating considerable heterogeneity in levels of expression. However, the distributions of fluorescence intensity of CHO cells stained with the TSK mAbs are all relatively narrow. Thus, although we do not know what molecule on the CHO cells reacts with the TSK antibodies, they do not react with the transfected $Fc_\gamma RII$ molecule.

To support this conclusion, we examined a mouse macrophage cell line, P388D1, transfected with $Fc_\gamma RII$. This cell line, PW16, expresses 1.2×10^6 $Fc_\gamma RII$ molecules/cell as determined by Scatchard analysis (data not shown), and also expresses murine $Fc_\gamma RII$ at $\sim 2.5 \times 10^5$ sites/cell. PW16 and the control P388D1 cells transfected with the insert in the opposite orientation, CPW00, show comparable fluorescence staining by TSK22 (Fig. 3). The staining of both the transfected and control P388D1 lines is inhibited substantially and to the same extent by mAb 2.4G2, which reacts specifically with mouse $Fc_\gamma RII$. There is, however, no inhibition of TSK22 binding after addition of mAb IV.3. These results are consistent with the results found for the $Fc_\gamma RII$-transfected CHO cells (Fig. 2), and show that the TSK mAbs do not react with human $Fc_\gamma RII$.

Enzyme Release. All the IgM antibodies specific for $Fc_\gamma R$ triggered neutrophil degranulation. We used β-gluc-

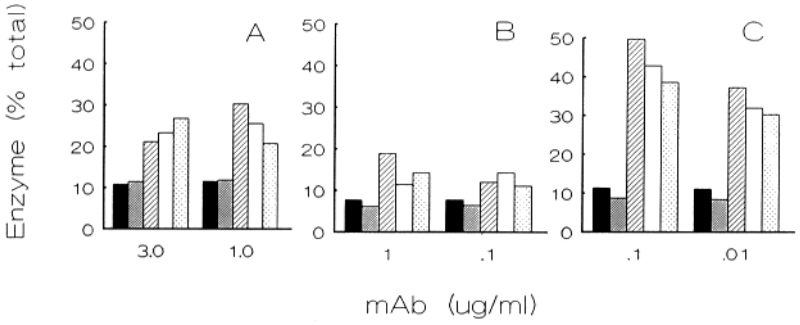

Figure 4. Release from neutrophils of hydrolytic enzymes after stimulation with IgM anti-FcγR mAbs. We measured the release of neutrophil elastase (*A*), β-glucuronidase (*B*), and alkaline phosphatase (*C*) from human neutrophils (10^6) incubated with TSK1 (□), TSK23 (▨), TSK26 (▩), mouse IgM (▨), or medium (■). Neutrophils lysed in 1% NP-40 were used to determine the total cellular enzyme content. Total enzyme activity of the supernatant is normalized as the percentage of total cellular enzyme content.

uronidase and neutrophil elastase as markers for azurophil granule release and alkaline phosphatase as a marker for specific granule release. The IgM anti-$Fc_\gamma R$ mAbs trigger degranulation very efficiently. Enzyme release over background levels was found at mAb concentrations as low as 0.01 μg/ml, (10^{-11} M) for alkaline phosphatase (Fig. 4 *C*). Normal mouse IgM, even at 10 μg/ml, did not trigger enzyme release over background at any time interval. The extent of degranulation was estimated by measuring the extent of enzyme release and normalizing by the total cellular enzyme present in NP-40 lysates of neutrophils. The release of neutrophil elastase was 25–45% of total (Fig. 4 *A*), β-glucuronidase was 10–25% of total (Fig. 4 *B*), and alkaline phosphatase was 30–50% of total (Fig. 4 *C*). The values are comparable with the release found after stimulation with FMLP, which at high concentrations is a potent stimulus of degranulation (36).

We found that mAbs 3G8 and IV.3 did not trigger degranulation (data not shown). These results are in agreement with others (37, 38), who found that there was no activation triggered by anti-$Fc_\gamma RII$ or $Fc_\gamma RIII$ mAbs unless the mAbs were crosslinked with anti-mouse IgG $F(ab')_2$. To determine the $Fc_\gamma R$ class on the neutrophil plasma membrane involved in triggering by the TSK anti-$Fc_\gamma R$ mAbs, we examined the effect of preincubating the cells with Fab fragments of mAbs 3G8 and IV.3. The cells were preincubated with both Fab fragments separately and in combination, and then the IgM anti-$Fc_\gamma R$ antibodies were added. There is dramatic inhibition in each enzyme assay with all three combinations of Fab fragments, even with IV.3 Fab alone (Fig. 5), thus demonstrating that in a functional assay, both $Fc_\gamma RII$ and $Fc_\gamma RIII$ are required.

Morphology. Given the extensive enzyme release triggered by the autoantibodies, we would expect to observe accompanying morphological changes. We examined neutrophils incubated with mAb TSK23 for 3 min and control cells incubated with normal mouse IgM by scanning electron microscopy. Averaged over 20 cells, the diameter of control neutrophils was greater (8.2 ± 1.3 μm) than that of neutrophils incubated with TSK23 (6.2 ± 0.6 μm) ($p < 0.01$). These results were confirmed by differential interference contrast microscopy (results not shown). The surface morphology of the cells is dramatically different in the presence of anti-$Fc_\gamma R$ antibody (Fig. 6). The cells treated with anti-$Fc_\gamma R$ antibody (Fig. 6, *C* and *D*) lack the large pseudopods and lamellae seen in control neutrophils (Fig. 6, *A* and *B*).

Transmission electron microscopy was carried to understand better the morphological changes occurring after stimulation with anti-$Fc_\gamma R$ antibodies from TSK mice. Relative to the control neutrophils (Fig. 7 *A*), the surface of the neutrophils incubated with TSK23 (Fig. 7 *B*) shows fewer membrane ruffles and infoldings, in agreement with the scanning results. The stimulated neutrophils have fewer large storage vesicles, and a much higher number of small vesicles than the control neutrophils. These results are consistent with extensive degranulation, and subsequent internalization of excessive membrane into smaller vesicles, resulting in a smaller cell volume.

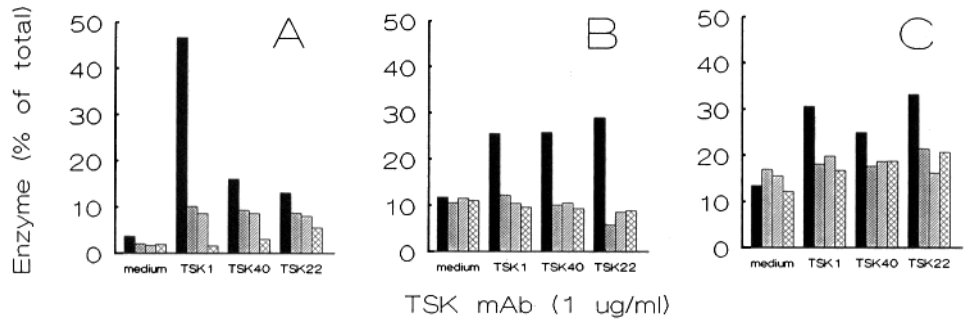

Figure 5. Effect of 3G8 Fab and IV.3 Fab on enzyme release of human neutrophils stimulated with IgM anti-$Fc_\gamma R$ mAbs. Neutrophils (10^6) were preincubated (4°C, 1 h) with Fab fragments (1 μg/ml), and then IgM anti-$Fc_\gamma R$ mAbs (1 μg/ml diluted in 10% (FCS/RPMI) were added for 30 min at 37°C. The neutrophil elastase (*A*), β-glucuronidase (*B*), and alkaline phosphatase (*C*) enzymatic activity in the supernatants is expressed as percent of total cellular enzyme content as in Fig. 4. Control (■), preincubation with 3G8 Fab (▩), preincubation with IV.3 Fab (▨), preincubation with 3G8 Fab + IV.3 Fab (▩).

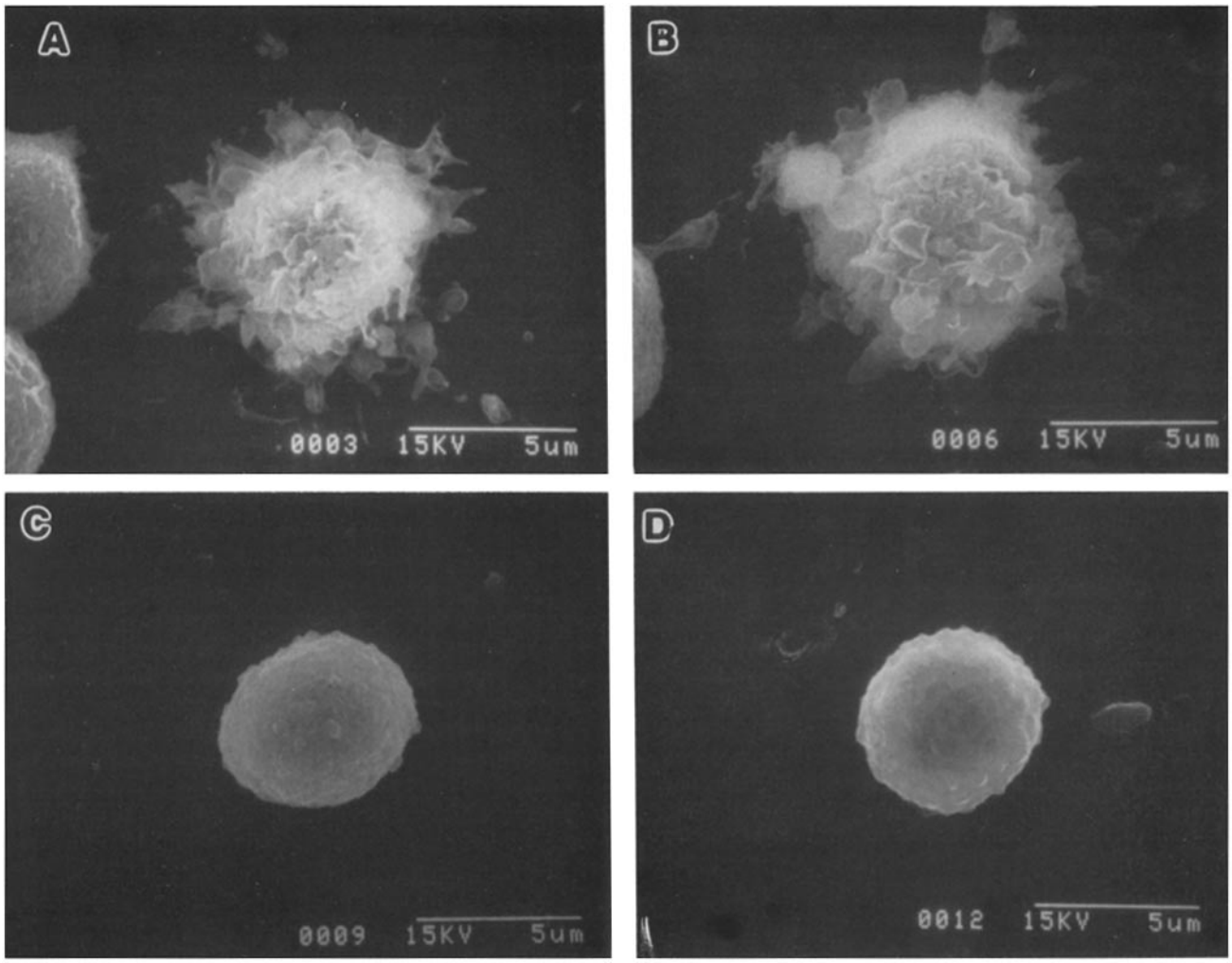

Figure 6. Scanning electron microscopy of human neutrophils (*A* and *B*) and neutrophils treated with TSK23 anti-$Fc_\gamma R$ mAb (*C* and *D*). Cells were incubated with the antibody (1 μg/ml) for 3 min. The bar is 5 μm (×5,000).

Discussion

Several inbred mouse strains used as models for autoimmune diseases, including NZB, NZB/NZW, TSK, and viable motheaten mice, have high circulating levels of IgM anti-mouse $Fc_\gamma RII$ autoantibody. In old NZB females, ~16 μg/ml, or 2%, of the total IgM bound to an $Fc_\gamma R$ affinity column (4). We have shown previously that sera from such mice, as well as IgM anti-$Fc_\gamma RII$ mAbs, inhibit macrophage $Fc_\gamma R$-mediated binding of immune complexes. These autoantibodies are probably responsible for the $Fc_\gamma R$ paralysis seen in peritoneal macrophages from autoimmune mice (5). It was thought that high levels of circulating immune complexes in autoimmune mice caused $Fc_\gamma R$ paralysis. In support of our hypothesis, peritoneal macrophages from BXSB male mice, which have no detectable anti-$Fc_\gamma R$ Ig but do have a severe lupus-like syndrome, are normal for macrophage $Fc_\gamma R$ function. Anti-$Fc_\gamma R$ autoantibodies have been detected in human disease as well, indicating juvenile neutropenia and SLE (6–8). We have preliminary evidence that such anti-$Fc_\gamma R$ antibodies are also present in human scleroderma patients (unpublished data).

We have found that the polyspecific anti-$Fc_\gamma R$ mAbs isolated from TSK mice bind with high avidity to human neutrophils, and that these autoantibodies efficiently trigger the release of azurophil and specific granule hydrolases. In contrast, mAbs directed against human $Fc_\gamma RIII$ or $Fc_\gamma RII$ do not induce hydrolase release without additional crosslinking (38, 39). The difference in efficacy of the anti-$Fc_\gamma R$ IgM compared with IgG mAbs may reflect the decavalent nature of IgM, which more efficiently clusters or crosslinks the receptors.

The anti-$Fc_\gamma R$ mAbs we isolated from the TSK mice, and the anti-$Fc_\gamma R$ mAbs previously characterized from NZB and me^v mice, were all polyspecific (4), binding to a wide range of cellular antigens including collagen type I and III, and dsDNA. The polyspecificity of the anti-$Fc_\gamma R$ mAbs may enhance the potential for possible pathogenicity. If the decavalent IgM autoantibody binds to collagen, for example, it could

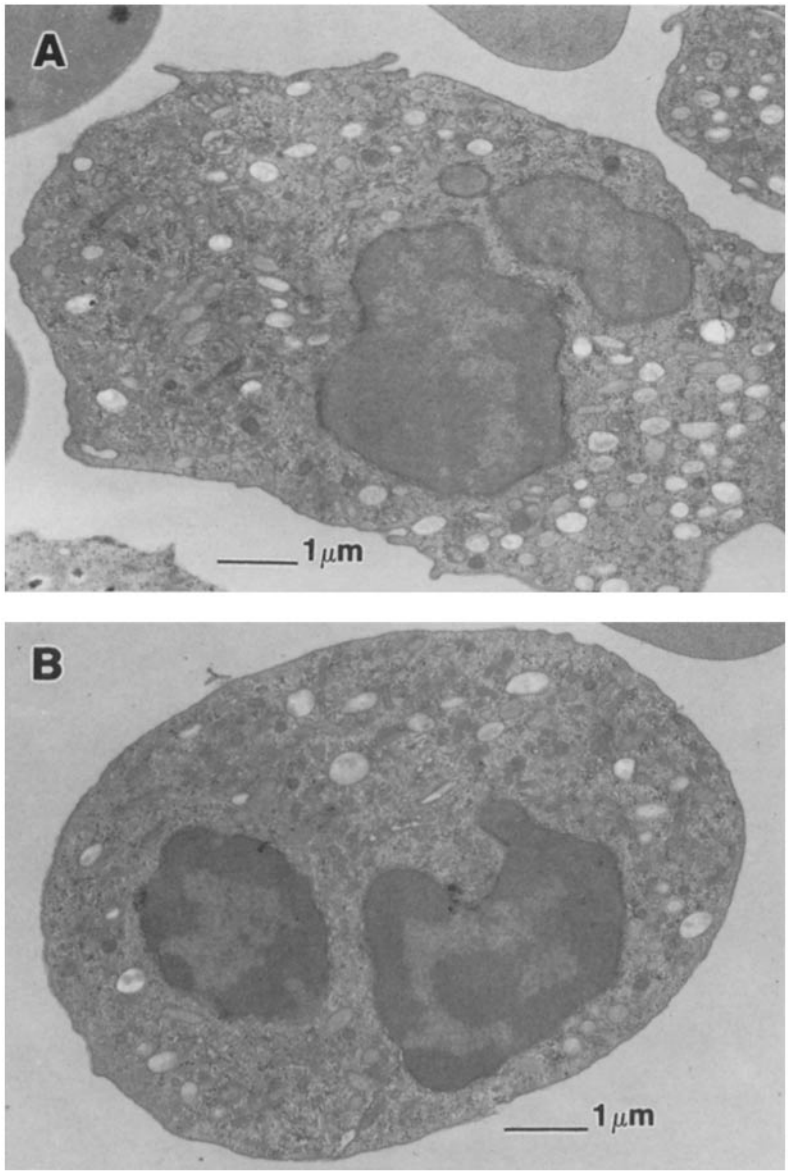

Figure 7. Transmission electron microscopy of human neutrophils (*A*) or neutrophils incubated with the anti-$Fc_\gamma R$ mAb TSK23 (*B*). Cells were incubated with TSK23 anti-$Fc_\gamma R$ (1 μg/ml) for 5 min. The bar is 1 μm (×11,200).

also bridge to the $Fc_\gamma R$ of neutrophils or macrophages, activating the release of chemotactic factors as well as hydrolytic enzymes in the immediate vicinity. Such an antibody might also play a role in activation of the mesangial cells of the kidney, which bear $Fc_\gamma Rs$ and can be triggered to release superoxide (40, 41). The role of polyspecific antibodies in autoimmune diseases is not well understood. Some suggest they are only marginally related to autoimmunity, constituting a natural network (42, 43). However it should be noted that anti-$Fc_\gamma R$ autoantibody is not found at detectable levels in normal mice (4).

In determining which $Fc_\gamma R$ subclass is responsible for degranulation by the IgM anti-$Fc_\gamma R$ mAbs, we found evidence that the signaling by the GPI-anchored $Fc_\gamma RIII$-1 requires human $Fc_\gamma RII$. The anti-$Fc_\gamma R$ IgM antibodies offer an advantage in analysis of the signalling requirements since they do not interact with $Fc_\gamma Rs$ via their Fc domains and they mediate triggering without the need for additional crosslinking. The dramatic enzyme release triggered by IgM anti-$Fc_\gamma R$ mAbs is inhibited by mAb 3G8 Fab (directed against $Fc_\gamma RIII$), and by mAb IV.3 Fab (directed against $Fc_\gamma RII$). The inhibition by mAb 3G8 is probably due to displacement of the IgM anti-$Fc_\gamma R$ autoantibody, and/or reduction of the extent of crosslinking below the threshold needed to trigger the neutrophil. The inhibition of neutrophil degranulation by the mAb IV.3 Fab is a particularly surprising result, given the lack of binding of the anti-$Fc_\gamma R$ mAbs from TSK mice to human $Fc_\gamma RII$. The results suggest that signal transduction may require $Fc_\gamma RII$ activation subsequent to crosslinking of the GPI-anchored $Fc_\gamma RIII$-1 molecule. This differs from Kimberly et al. (38), who suggest that $Fc_\gamma RIII$ alone, crosslinked with anti-$Fc_\gamma RIII$ Fab and anti–mouse IgG

$F(ab')_2$, can trigger $[Ca^{2+}]_i$ flux. The same group finds that Con A–opsonized E also are phagocytosed via $Fc_\gamma RIII$, which bears high mannose oligosaccharides (44).

Signal transduction via neutrophil $Fc_\gamma RIII$-1 may resemble that of other GPI-anchored molecules, such as Thy-1 (45, 46), the Ly-6-encoded molecule T cell activating protein (TAP) (47, 48), and 5'-nucleotidase (CD73) (49, 50). Crosslinking of Thy-1 and TAP results in T cell mitogenesis only in the presence of a functional CD3/Ti complex. Furthermore, the GPI anchor is required for signalling through TAP. Signalling via GPI-anchored proteins also differs fundamentally from the Ti/CD3 complex in that anti-TAP mAbs do not trigger T cell mitogenesis when adsorbed to surfaces, but only in solution (51).

Therefore, there is precedent for the hypothesis that anti-$Fc_\gamma R$ IgM mAb clustering of neutrophil $Fc_\gamma RIII$-1 triggers signalling by activating $Fc_\gamma RII$. For crosslinking of $Fc_\gamma RIII$-1 to trigger $Fc_\gamma RII$, one must postulate interaction of ectodomains of the receptors, and/or interaction involving the GPI anchor. The latter is suggested by experiments demonstrating that the GPI anchor is required for TAP triggering (48). However, the inhibition by the anti-human $Fc_\gamma RII$ mAb Fab fragment of enzyme release triggered by the IgM anti-$Fc_\gamma R$ mAb suggests that interaction of the ectodomains is required as well. The phospholipid anchor of erythrocyte acetylcholinesterase has been determined to consist of an 18:0 or 18:1 1-alkyl group and unusual unsaturated 22:4 or 22:5 2-acyl groups (52). A microdomain in the plasma membrane consisting exclusively of such phospholipid anchors might have unique properties that would interact with other signalling proteins.

Our results clearly show that the IgM anti-$Fc_\gamma R$ mAbs, which are a subset of the polyspecific mAbs found in TSK mice, mediate a dramatic degranulation of human neutrophils. The release of hydrolytic enzymes occurs at extremely low concentrations of IgM mAb, and may be important in inflammation accompanying autoimmunity.

We thank Andrew Pizzimenti for assistance with the flow cytometer analysis.

This work was supported by National Institutes of Health grants AI-24322 and AI-24671. Sandra K. Masur is an Irma T. Hirschl Career Scientist.

Address correspondence to Jay Unkeless, Department of Biochemistry, Box 1020, Mount Sinai School of Medicine, 1 Gustave Levy Place, New York City, NY 10029.

Received for publication 17 December 1990 and in revised form 19 February 1991.

References

1. Anegon, I., M.C. Cuturi, G. Trinchieri, and B. Perussia. 1988. Interaction of Fc receptor (CD16) ligands induces transcription of interleukin 2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J. Exp. Med.* 167:452.
2. Odin, J.A., C.J. Painter, and J.C. Unkeless. 1990. Fc gamma receptors: a diverse and multifunctional gene family. *In* Receptors of Inflammatory Cells: Structure-Function Relationships (Volume 1 of Cellular and Molecular Mechanisms of Inflammation). C.G. Cochrane and M.A. Gimbrone, Jr., editors. Academic Press, Inc., Orlando. 1–33.
3. Almon, R.R., C.G. Andrew, and S.H. Appel. 1974. Serum globulin in myasthenia gravis: inhibition of alpha-bungarotoxin binding to acetylcholine receptors. *Science (Wash. DC).* 186:55.
4. Boros, P., J. Chen, C. Bona, and J.C. Unkeless. 1990. Autoimmune mice make anti-Fc γ receptor Ig. *J. Exp. Med.* 171:1581.
5. Russell, P.J., and A.D. Steinberg. 1983. Studies of peritoneal macrophage function in mice with systemic lupus erythematosus: depressed phagocytosis of opsonized sheep erythrocytes in vitro. *Clin. Immunol. Immunopathol.* 27:387.
6. Lalezari, P., M. Khorshidi, and M. Petrosova. 1986. Autoimmune neutropenia of infancy. *J. Pediatr.* 109:764.
7. Madyastha, P.R., H.H. Fudenberg, A.B. Glassman, K.R. Madyastha, and C.L. Smith. 1982. Autoimmune neutropenia in early infancy: a review. *Ann. Clin. Lab. Sci.* 12:356.
8. Sipos, A., C. Csortos, S. Sipka, P. Gergely, I. Sonkoly, and G. Szegedi. 1988. The antigen/receptor specificity of antigranulocyte antibodies in patients with SLE. *Immunol. Lett.* 19:329.
9. Frank, M.M., T.J. Lawley, M.I. Hamburger, and E.J. Brown. 1983. NIH Conference: immunoglobulin G Fc receptor-mediated clearance in autoimmune diseases. *Ann. Intern. Med.* 98:206.
10. Kastori, K., M. Monestier, R. Mayer, and C. Bona. 1988. Biased usage of certain Vk gene families by autoantibodies and their polymorphism in autoimmune mice. *Mol. Immunol.* 25:213.
11. Muryoi, T., K.N. Kasturi, M.J. Kafina, Y. Saitoh, O. Usuba, J.S. Perlish, R. Fleischmajer, and C.A. Bona. 1991. Self reactive repertoire of tight skin mouse: immunochemical and molecular characterization of anti-topoisomerase I autoantibodies. *Autoimmunity.* In press.
12. Qu, Z., J. Odin, J.D. Glass, and J.C. Unkeless. 1988. Expression and characterization of a truncated murine Fcγ receptor. *J. Exp. Med.* 167:1195.
13. English, D., and B.R. Anderson. 1974. Single step separation of red blood cells, granulocytes, and mononuclear leukocytes on discontinuous density gradient of Ficoll-Hypaque. *J. Immunol. Methods.* 5:249.

14. Hibbs, M.L., L. Bonadonna, B.M. Scott, I.F. McKenzie, and P.M. Hogarth. 1988. Molecular cloning of a human immunoglobulin G Fc receptor. *Proc. Natl. Acad. Sci. USA.* 85:2240.
15. Brooks, D.G., W.Q. Qiu, A.D. Luster, and J.V. Ravetch. 1989. Structure and expression of human IgG FcRIII(CD32). Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *J. Exp. Med.* 170:1369.
16. Miller, J., T.R. Malek, W.J. Leonard, W.C. Greene, E.M. Shevach, and R.N. Germain. 1985. Nucleotide sequence and expression of a mouse interleukin 2 receptor cDNA. *J. Immunol.* 134:4212.
17. Maniatis, T., E.F. Frisch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 76:1373.
19. Mitchell, P.J., A.M. Carothers, J.H. Han, J.D. Harding, E. Kas, L. Venolia, and L.A. Chasin. 1986. Multiple transcription start sites, DNase I-hypersensitive sites, and an opposite-strand exon in the 5′ region of the CHO DHFR gene. *Mol. Cell. Biol.* 6:425.
20. Gunning, P., J. Leavitt, G. Muscat, S. Ng, and L. Kedes. 1987. A human beta-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA.* 84:4831.
21. Hirata, Y., T. Taga, M. Hibi, N. Nakano, T. Hirano, and T. Kishimoto. 1989. Characterization of IL-6 receptor expression by monoclonal and polyclonal antibodies. *J. Immunol.* 143:2900.
22. Goding, J.W. 1990. Conjugation of antibodies with fluorochromes: Modification of the standard methods. *J. Immunol. Methods.* 1976:215.
23. Fleit, H.B., S.D. Wright, and J.C. Unkeless. 1982. Human neutrophil Fc gamma receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79:3275.
24. Ceuppens, J.L., F.L. Bloemman, and J.P. Van Wauwe. 1985. T cell unresponsiveness to the mitogenic activity of OKT3 antibody results from a deficiency of monocyte Fc-gamma receptors for murine IgG2a and inability to crosslink the T3-Ti complex. *J. Immunol.* 135:3882.
25. Harper, W., R.R. Cook, J. Roberts, B.J. McLaughlin, and J.C. Powers. 1984. Active site mapping of the serine proteases human leukocyte elastase, cathepsin G, porcine pancreatic elastase, rat mast cell proteases I and II, bovine chymotrypsin alpha, and *Staphylococcus aureus* protease V-8 using tripeptide thiobenzyl substrates. *Biochemistry.* 23:2995.
26. Hausamen, T.U., R. Helger, W. Rick, and W. Gross. 1967. Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method. *Clin. Chim. Acta.* 15:241.
27. Karnovsky, M.J. 1971. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. American Society for Cell Biology Abstracts. 11th Annual Meeting.
28. Watson, M.L. 1958. Staining of tissue sections for EM with heavy metals. *J. Biophys. Biochem. Cytol.* 4:475.
29. Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208.
30. Jimenez, S.A., A. Millan, and R.I. Bashey. 1984. Scleroderma-like alterations in collagen metabolism occurring in the TSK (tight skin) mouse. *Arthritis Rheum.* 27:180.
31. Jimenez, S.A., C.J. Williams, J.C. Meyers, and R.I. Bashey. 1986. Increased collagen biosynthesis and increased expression of type I and type III procollagen genes in tight skin (TSK) mouse fibroblasts. *J. Biol. Chem.* 261:657.
32. Douval, A.S., M. Achten, and E.M. Tan. 1979. Identification of nuclear protein (Scl 70) as a unique target of human antinuclear antibodies in scleroderma. *J. Biol. Chem.* 254:10514.
33. Petroni, K.C., L. Shen, and P.M. Guyre. 1988. Modulation of human polymorphonuclear leukocyte IgG Fc receptors and Fc receptor-mediated functions by IFN-gamma and glucocorticoids. *J. Immunol.* 140:3467.
34. Tosi, M.F., and M.F. Berger. 1988. Functional differences between the 40 kDa and 50–70 kDa IgG Fc receptors on human neutrophils revealed by elastase treatment and anti-receptor antibodies. *J. Immunol.* 141:2097.
35. Huizinga, T.W.J., M. Kerst, J.H. Nuyens, A. Vlug, A.E. von dem Borne, D. Roos, and P.A.T. Tetteroo. 1989. Binding characteristics of dimeric IgG complexes to human neutrophils. *J. Immunol.* 142:2359.
36. Glinski, W., M. Tigalonowa, S. Jablonska, and E. Janczura. 1986. Decreased extracellular release of granule enzymes from in vitro-stimulated polymorphonuclear leukocytes in guttate psoriasis. *Inflammation.* 10:99.
37. Huizinga, T.W., K.M. Dolman, N.J. van der Linden, M. Kleijer, J.H. Nuijens, A.E. von dem Borne, and D. Roos. 1990. Phosphatidylinositol-linked FcRIII mediates exocytosis of neutrophil granule proteins, but does not mediate initiation of the respiratory burst. *J. Immunol.* 144:1432.
38. Kimberly, R.P., J.W. Ahlstrom, M.E. Click, and J.C. Edberg. 1990. The glycosyl phosphatidylinositol-linked $Fc\gamma RIII_{PMN}$ mediates transmembrane signaling events distinct from $Fc_\gamma RII$. *J. Exp. Med.* 171:1239.
39. Gresham, H.D., A. Zheleznyak, J.S. Mormol, and E.J. Brown. 1990. Studies on the molecular mechanisms of human neutrophil Fc receptor-mediated phagocytosis. Evidence that a distinct pathway for activation of the respiratory burst results in reactive oxygen metabolite-dependent amplification of ingestion. *J. Biol. Chem.* 265:7819.
40. Neuwirth, R., P. Singhal, B. Diamond, R.M. Hays, L. Lobmeyer, K. Clay, and D. Schlondorff. 1988. Evidence for immunoglobulin Fc receptor-mediated and platelet-activating factor formation by cultured rat mesangial cells. *J. Clin. Invest.* 82:936.
41. Santiago, A., J. Satriano, S. DeCandido, H. Holthofer, R. Schreiber, J. Unkeless, and D. Schlondorff. 1989. A specific Fc gamma receptor on cultured rat mesangial cells. *J. Immunol.* 143:2575.
42. Avrameas, S., B. Guibert, W. Mahana, P. Matsiota, and T. Ternynck. 1988. Recognition of self and non-self constituents by polyspecific autoreceptors. *Int. Rev. Immunol.* 3:1.
43. Schwartz, R.S. 1988. Polyvalent anti-DNA autoantibodies: Immunochemical and biological significance. *Int. Rev. Immunol.* 3:97.
44. Salmon, J.E., S. Kapur, and R.P. Kimberly. 1987. Opsonin-independent ligation of Fc gamma receptors. The 3G8-bearing receptors on neutrophils mediate the phagocytosis of concanavalin A-treated erythrocytes and nonopsonized *Escherichia coli*. *J. Exp. Med.* 166:1798.
45. Schmitt-Verhulst, A.M., A. Guimezanes, C. Boyer, M. Poenie, R. Tsien, M. Buferne, C. Hua, and L. Leserman. 1987. Pleitropic loss of activation pathways in a T-cell receptor alpha chain deletion variant of a cytolytic T cell clone. *Nature (Lond.).* 325:628.
46. Gunter, K.C., R.N. Germain, R.A. Kroczek, T. Saito, W.M. Yokoyama, C. Chan, A. Weiss, and E.M. Shevach. 1987. Thy-1 mediated T-cell activation requires coexpression of CD3/Ti

complex. *Nature (Lond.).* 326:505.
47. Bamezai, A., H. Reiser, and K.L. Rock. 1988. T cell receptor/CD3 negative variants are unresponsive to stimulation through the Ly-6 encoded molecule, TAP. *J. Immunol.* 141:1423.
48. Yeh, E.T., H. Reiser, A. Bamezai, and K.L. Rock. 1988. TAP transcription and phosphatidylinositol linkage mutants are defective in activation through the T cell receptor. *Cell.* 52:665.
49. Thomson, L.F., J.M. Ruedi, A. Glass, G. Moldenhauer, P. Moller, M.G. Low, M.R. Klemens, M. Massaia, and A.H. Lucas. 1990. Production and characterization of monoclonal antibodies to the glycosyl phosphatidylinositol-anchored lymphocyte differentiation antigen ecto-5'-nucleotidase (CD73). *Tissue Antigens.* 35:9.
50. Thompson, L.F., J.M. Ruedi, A. Glass, M.G. Low, and A.H. Lucas. 1989. Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. *J. Immunol.* 143:1815.
51. Bamezai, A., V. Goldmacher, H. Reiser, and K.L. Rock. 1989. Internalization of phosphatidylinositol-anchored lymphocyte proteins. I. Documentation and potential significance for T cell stimulation. *J. Immunol.* 143:3107.
52. Roberts, W.L., J.J. Myher, A. Kuksis, M.G. Low, and T.L. Rosenberry. 1988. Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. Palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. *J. Biol. Chem.* 263:18766.