

# Neutrophil-binding Immunoglobulin G in Systemic Lupus Erythematosus

## EVIDENCE FOR PRESENCE OF BOTH SOLUBLE IMMUNE COMPLEXES AND IMMUNOGLOBULIN G ANTIBODIES TO NEUTROPHILS

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**ABSTRACT** The objectives of these studies were to quantify the amounts of immunoglobulin (Ig)G bound to peripheral blood neutrophils from patients with systemic lupus erythematosus (SLE) and to determine the contributions of soluble immune complexes or anticell antibodies to the levels of IgG neutrophil-binding activity in SLE sera.

Neutrophil-bound IgG, determined by a sensitive antiglobulin inhibition assay, was elevated in 7 out of 14 SLE patients compared with values obtained in 23 normal controls. The levels of IgG neutrophil-binding activity in sera were elevated in 22 of 38 patients with SLE over the values seen with 36 normal sera. No correlation was found between the peripheral blood neutrophil counts in the SLE patients and the values for IgG adherent to the cells or serum cell-binding activity. The sera from 18 patients with SLE were fractionated by gel filtration. Elevated levels of IgG neutrophil-binding activity were found in 11 of the 18 G-200 excluded pools and in 13 of the G-200 IgG pools. In nine sera elevated levels were observed in both pools. F(ab')<sub>2</sub> fragments of IgG from SLE sera bound to normal polymorphonuclear leukocytes in greater amounts than F(ab')<sub>2</sub> fragments of IgG from normal sera. A significant correlation existed between the values of IgG neutrophil-binding activity found in SLE sera and those obtained with both the G-200 excluded and IgG pools. Sucrose density gradient fractionation of four sera from SLE patients confirmed the presence of both large (>19S) and intermediate-sized (7S-19S) cell-binding immune complexes as well as of

monomeric IgG antibodies to neutrophils. The levels of IgG neutrophil-binding activity in the SLE sera correlated well with the results obtained with the Raji cell assay for immune complexes as well as with the titer of antibodies to nuclear antigens.

These data indicate that circulating neutrophils from patients with SLE commonly have increased amounts of cell-bound IgG. The elevated levels of IgG neutrophil-binding activity in the sera of these patients are caused by both soluble immune complexes and antibodies reactive with neutrophils.

## INTRODUCTION

Increased amounts of immunoglobulin (Ig)G on or within circulating polymorphonuclear leukocytes (PMN)<sup>1</sup> from patients with systemic lupus erythematosus (SLE) have been demonstrated previously using qualitative techniques. Unfractionated leukocytes from SLE patients were shown to possess enhanced levels of IgG using an indirect antiglobulin consumption test (1). Intracellular inclusions of immunoglobulins were demonstrated in PMN from SLE patients by immunofluorescence techniques (2). Incubation of SLE sera with normal PMN at 37°C in vitro produced immunoglobulin-containing inclusions within the cells (3). These results have been interpreted as indicating that soluble immune complexes are adherent to and ingested by circulating PMN in

<sup>1</sup> *Abbreviations used in this paper:* ANA, antibodies to nuclear antigens; IgG-PBA, IgG-PMN binding activity; NRS, normal rabbit serum; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte(s); SAS, saturated ammonium sulfate; SDG, sucrose density gradient; SLE, systemic lupus erythematosus.

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vivo in SLE patients. Also, normal PMN have been used to assay for the presence of soluble immune complexes in various sera (4). The possible contribution of anti-PMN antibodies, however, to the cell-bound immunoglobulins in SLE and other disorders has not been investigated.

A precise quantification of cell-bound IgG can be obtained using recent adaptations of the antiglobulin consumption assay. Cases of Felty's syndrome have been studied using this technique with the demonstration of increased amounts of IgG bound to the patients' own PMN (5). Elevated serum levels of PMN-binding IgG have been observed in a patient with thyroiditis and neutropenia (6). These studies did not determine, however, whether the neutrophil-associated IgG or serum IgG-PMN binding activity in the described patients was caused by immune complexes or by antibodies to neutrophils.

We have used an antiglobulin consumption technique to quantify the amounts of IgG adherent to SLE or normal PMN. The IgG-PMN binding activity (IgG-PBA) in serum, or serum fractions, also was determined. The results indicate that PMN from SLE patients possess twice the normal amount of cell-bound IgG. Increased levels of IgG-PBA were detected in SLE sera and in serum fractions containing either immune complexes or monomeric IgG. The levels of IgG-PBA in sera, however, correlated best with those in the IgG fractions. These results indicate that although immune complexes may bind to PMN in vivo in SLE patients, antibodies to PMN may contribute as well to the cell-associated IgG.

## METHODS

**Blood samples.** Normal control sera and purified neutrophils were obtained from healthy laboratory workers and hospital staff at the Seattle Veterans Administration Hospital. Blood samples were obtained from patients with SLE who were seen at one of the University of Washington-affiliated hospitals or in the offices of practicing rheumatologists in Seattle. All patients fulfilled the American Rheumatism Association criteria for SLE (7). Neutrophils were purified from 14 SLE patients, and sera were obtained from 38 patients. Serum samples were also obtained from six patients with IgG myeloma or benign IgG monoclonal gammopathy through the courtesy of Dr. Bruce C. Gilliland (University of Washington School of Medicine, Seattle, Wash.). The subclasses of the IgG paraproteins were kindly determined by Dr. John Leddy (University of Rochester School of Medicine, Rochester, N. Y.). All sera were heat-inactivated at 56°C for 30 min. The sera were then kept at 4°C for 3 d, centrifuged to remove possible cryoglobulins, and were stored in aliquots at -20°C until assayed. After thawing, each serum sample was centrifuged in a Beckman Model B microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 3 min at 4°C immediately before incubation with PMN.

**Purification of PMN.** PMN were obtained from fresh whole blood, anticoagulated with EDTA, by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) gradients followed by sedimentation of erythrocytes

with 3% dextran, molecular weight 500,000 (Sigma Chemical Co., St. Louis, Mo.), as described (8, 9). Residual erythrocytes were lysed by brief hypotonic shock with 0.2% NaCl. These preparations contained 94-98% granulocytes, which were >95% viable by trypan blue dye exclusion. After two further washings with phosphate-buffered saline (PBS) for a total of six washes, the PMN were suspended in 10 mM 2-deoxyglucose in PBS to inhibit interiorization of membrane-bound IgG (10) and were counted in a clinical hemacytometer in quadruplicate. No erythrocyte membrane fragments were present in Wright's-stained PMN preparations.

**Fab anti-F(ab')<sub>2</sub> assay.** This assay, described in detail (9, 11), is a quantitative antiglobulin consumption technique that uses the marked solubility difference in 50% saturated ammonium sulfate (SAS) of unbound human <sup>125</sup>I-Fab compared to <sup>125</sup>I-Fab bound to rabbit antihuman F(ab')<sub>2</sub>. Human IgG was purified from commercial Cohn fraction II (Schwartz/Mann Div., Beckton, Dickinson & Co., Orangeburg, N. Y.) by ion exchange and gel filtration chromatography. The F(ab')<sub>2</sub> fragments of IgG were obtained by pepsin digestion followed by gel filtration on Sephadex G-150 (Pharmacia Fine Chemicals) (12). The Fab fragments of IgG were obtained by papain digestion followed by sequential ion exchange chromatography on CM cellulose and DEAE cellulose (13). After gel filtration on Sephadex G-100, this preparation contained only Fab fragments as determined by double diffusion studies in agar using antisera specific for human Fab or Fc. Radioiodination of the Fab fragments was performed by the iodine monochloride technique (14).

Antisera to F(ab')<sub>2</sub> were raised in rabbits that were bled after 3 mo of biweekly boosting with F(ab')<sub>2</sub> in complete Freund's adjuvant. The titer of anti-F(ab')<sub>2</sub>, which bound 50% of 20 ng <sup>125</sup>I-Fab, was determined by precipitation in 50% SAS as described (11). 0.5-ml aliquots of serial dilutions of antiserum with PBS containing 1% normal rabbit serum (NRS) were incubated with 20 ng <sup>125</sup>I-Fab suspended in 0.5 ml PBS containing 10% NRS for 18 h at 4°C. 1 ml of ice-cold SAS was added with stirring. The mixture was allowed to sit at 4°C for 30 min, then was centrifuged at 2,500 g for 30 min at 4°C. The precipitate was washed twice with 50% SAS and was counted in a well-type gamma counter. The percentage of <sup>125</sup>I-Fab precipitated by the rabbit anti-F(ab')<sub>2</sub> antibodies (%P) was calculated by the formula: %P = 100 (1-[counts per minute A-counts per minute B]/[counts per minute A-counts per minute C]), where A = 20 ng <sup>125</sup>I-Fab, B = the <sup>125</sup>I-Fab bound to anti-F(ab')<sub>2</sub> (i.e., precipitated in 50% SAS), and C = <sup>125</sup>I-Fab in PBS with 10% NRS precipitated nonspecifically in 50% SAS.

The dilution of anti-F(ab')<sub>2</sub> antiserum that bound 50% of 20 ng <sup>125</sup>I-Fab was calculated and aliquots of the dilution were used to obtain a standard inhibition curve with 1.5-100 ng monomeric human IgG as follows. On day 1, the IgG in 0.5 ml of PBS with 1% NRS was incubated with the aliquot of anti-F(ab')<sub>2</sub> in 0.5 ml PBS with 1% NRS at 4°C. On day 2, 20 ng <sup>125</sup>I-Fab was added as before. On day 3, 1.5 ml of SAS was added, and the amount of <sup>125</sup>I-Fab in the precipitate was determined as before. The IgG added to the antiserum inhibited subsequent binding of the <sup>125</sup>I-Fab. A standard inhibition curve was obtained by plotting the percentage of antibody-bound Fab (%P) vs. the logarithm of the amount of IgG added. 12-fold greater amounts of purified human monoclonal IgM or IgA than human IgG were necessary to produce comparable inhibition, as was found by others (15).

**PMN-bound IgG (direct assay).** Serial twofold dilutions of the purified PMN suspension containing 2 × 10<sup>6</sup>-1.25 × 10<sup>5</sup> cells were lysed by freezing and thawing three times and the IgG in the suspension was determined by the Fab anti-F(ab')<sub>2</sub> assay. The amounts of inhibition of binding of anti-F(ab')<sub>2</sub> to <sup>125</sup>I-Fab produced by the serial dilutions of lysed

PMN were extrapolated to the linear, midportion of the standard curve to calculate the nanograms of IgG bound per  $10^6$  PMN. The antiserum did not bind nonspecifically to cell protein, since twofold greater numbers of lysed rabbit PMN caused no inhibition of binding of anti-F(ab')<sub>2</sub> to <sup>125</sup>I-Fab, as was also found by Smith et al. (11). The standard curve was repeated with every assay and at least two points on the standard curve were obtained for every test sample.

**IgG-PBA of serum (indirect assay).** 5 million PMN obtained from a normal donor by techniques outlined above were suspended in 100  $\mu$ l of patient or normal serum at room temperature for 30 min. After six washes with 5 ml of ice-cold PBS containing 1 mM EDTA, the cells were suspended in 1 ml of PBS with 1% NRS and were recounted. After lysing the PMN by freezing and thawing, the amount of IgG in the suspension was quantitated by the Fab anti-F(ab')<sub>2</sub> assay as outlined above. Controls in every experiment included donor PMN both with and without incubation in normal human serum.

**Serum fractionation.** Normal and SLE serum samples were fractionated by gel filtration on a Sephadex G-200 column (2.5  $\times$  100 cm) equilibrated with PBS at 4°C. 1.5 ml of serum was passed over the column, and pools of the excluded, IgG and albumin peaks were collected and concentrated to 1.5 ml by ultrafiltration on Millipore membranes (Millipore Corp., Bedford, Mass.) or by vacuum dialysis. The IgG concentration in the IgG pool was quantitated using radial immunodiffusion test plates (Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif.), and the protein concentration in the excluded pool was determined by the method of Lowry et al. (16) with human serum albumin as the standard. 200  $\mu$ l of each concentrated pool, containing 2.0  $\mu$ l of NRS to reduce nonspecific binding of IgG to PMN, were incubated with  $5 \times 10^6$  normal PMN exactly as described for serum. After six washes with cold PBS containing 1 mM EDTA, the cells were recounted, lysed, and the amount of IgG bound to PMN was determined using the Fab anti-F(ab')<sub>2</sub> assay as described above.

1 ml of normal or SLE serum samples were also fractionated by linear sucrose density gradient (SDG) ultracentrifugation, as described (9). The gradients were harvested and five approximately equal pools were made containing material >19S, 19S, 7S–19S, 7S, and <7S in size. After dialysis against PBS, each pool was concentrated to an equal volume so that the IgG in the 7S fraction was the same as the original serum IgG concentration. 100  $\mu$ l of each pool, containing 1.0  $\mu$ l of NRS, were incubated with  $5 \times 10^6$  PMN. After six washes, the PMN-bound IgG was quantitated as before.

**Pepsin digestion of normal or SLE IgG.** The F(ab')<sub>2</sub> fragments of purified IgG from normal or SLE sera were prepared by pepsin digestion (12). IgG fractions were obtained from serum either by gel filtration on G-200 or by ion exchange chromatography on DEAE cellulose equilibrated with 0.01 M phosphate buffer, pH 7.4. After pepsin digestion of IgG, the F(ab')<sub>2</sub> fragments were separated by gel filtration on Sephadex G-150 and concentrated to 8 mg/ml. For some studies, pepsin agglutinators (17) were removed by incubating the F(ab')<sub>2</sub> fragments with an equal volume of Sepharose 4B-linked normal human F(ab')<sub>2</sub> at room temperature for 2 h. The suspension was then centrifuged and the supernate containing F(ab')<sub>2</sub> was decanted. The amount of binding of F(ab')<sub>2</sub> fragments of IgG from normal or SLE sera to normal PMN was determined as for whole IgG using a standard curve prepared with normal human F(ab')<sub>2</sub> (IgG from Cohn fraction II).

**Immunoabsorption of rabbit antihuman F(ab')<sub>2</sub>.** For some experiments, the rabbit antihuman F(ab')<sub>2</sub> antiserum was made highly specific for the Fd portion of IgG. Purified

human monoclonal IgM $\kappa$ , IgA $\kappa$ , and IgA $\lambda$  paraproteins were separately coupled to Sepharose 4B (Pharmacia Fine Chemicals). The gels were equilibrated with borate buffer and were used as solid-phase immunoabsorbents (18). After sequentially passing an aliquot of anti-F(ab')<sub>2</sub> over each immunoabsorbent column, the effluent was concentrated to the original volume. The titer of adsorbed anti-F(ab')<sub>2</sub> that bound 50% of 20 ng <sup>125</sup>I-Fab was 1:600, compared to a titer of 1:5,000 for the unadsorbed antiserum. The adsorbed anti-F(ab')<sub>2</sub> antiserum gave no lines against purified human IgM and IgA paraproteins, or against purified  $\kappa$ - and  $\lambda$ -light chains by double diffusion studies in agar. Furthermore, amounts of IgM or IgA over 200-fold greater than that of IgG were necessary to produce comparable degrees of inhibition of binding of the adsorbed antiserum to <sup>125</sup>I-Fab.

**Heat aggregation of normal human IgG.** Human IgG was purified from commercial Cohn fraction II by ion exchange chromatography on DEAE cellulose. After concentration to 20 mg/ml and dialysis against PBS, the IgG was aggregated by heating at 63°C for 11 min. The size distribution of aggregated IgG in the sample was determined by linear SDG ultracentrifugation. Increasing amounts of aggregated IgG were added to PBS or to aliquots of normal serum, both fresh and heat inactivated at 56°C for 30 min. The levels of IgG-PBA in the samples were then determined as outlined above by incubating the serum or PBS containing aggregates with normal PMN.

**Immunofluorescent staining of PMN.** Normal PMN were incubated with seven normal or nine SLE sera, or with the G-200 IgG pools from these sera, and then washed six times exactly as described for the indirect assay for IgG-PBA in serum. 1 million IgG-coated PMN were then incubated with 25  $\mu$ l of fluorescein isothiocyanate-labeled F(ab')<sub>2</sub> fragments of goat antihuman F(ab')<sub>2</sub> (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) for 60 min at 0°C. After three washes with ice-cold PBS, the PMN were examined immediately for immunofluorescence using a Zeiss epi-illuminated fluorescent microscope (Carl Zeiss, Inc., New York). Controls consisted of PMN incubated only with PBS before adding the fluorescent antiserum.

**Other methods.** Antibodies to nuclear antigens (ANA) were determined in the laboratory of Dr. Bruce Gilliland using described techniques (19). The Raji cell assay was kindly carried out by Dr. Argyrios Theofilopoulos (Scripps Clinic and Research Foundation, La Jolla, Calif.) (20). The opsonization assay for anti-PMN antibodies was performed in our laboratory as recently described (9).

Testing for significant differences between groups was performed using Student's *t* test. Correlations between paired values were tested for significant differences using standard tables. The results of the ANA titers were tested using Spearman's rank correlation method (21).

## RESULTS

**PMN-bound IgG (direct assay).** The amounts of IgG bound to neutrophils from 23 normal individuals and from 14 patients with SLE were determined by the Fab anti-F(ab')<sub>2</sub> technique. The mean value ( $\pm$ 1 SD) for the normal cells was  $13.7 \pm 4.7$  ng IgG/ $10^6$  PMN, as recently published (9). The mean ( $\pm$ 1 SD) for the SLE patients,  $26.0 \pm 11.4$  ng IgG/ $10^6$  PMN, was significantly greater than the mean for normals, ( $P < 0.001$ , Student's *t* test) (Fig. 1). 50% of the SLE patients (7 of 14) exhibited levels of PMN-bound IgG that were

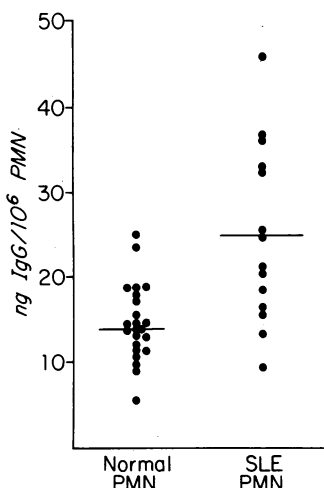


FIGURE 1 Neutrophil-bound IgG (ng IgG/ $10^6$  PMN) on cells purified from 23 normal controls and from 14 patients with SLE. The mean value for the SLE patients (horizontal bar) was significantly elevated over the mean for normals ( $P < 0.001$ , Student's  $t$  test).

elevated more than 2 SD above the normal mean. There was no correlation, however, between the blood neutrophil counts and the amounts of IgG adherent to the SLE patients' own PMN ( $r = 0.04$ ,  $P > 0.5$ ).

**Effect of heating or adding aggregated IgG to normal serum.** Because all of the SLE and normal sera were heated to inactivate complement, preliminary studies were performed to determine the effect of heating on serum levels of IgG-PBA. The amounts of IgG that bound to normal PMN after in vitro incubation with fresh, or heat-inactivated, SLE or normal sera were determined. The mean level of IgG-PBA of 10 unheated SLE sera was  $51.3 \pm 21.4$  ng IgG/ $10^6$  PMN compared to a mean of  $23.3 \pm 3.2$  ng IgG/ $10^6$  PMN for 7 normal sera ( $P < 0.005$ , Student's  $t$  test). After heating, the mean IgG-PBA of the SLE sera increased by 36% to  $70.0 \pm 20.6$  ng IgG/ $10^6$  PMN, whereas the normal sera IgG-PBA increased 57% to  $36.5 \pm 2.2$  ng IgG/ $10^6$  PMN. These data indicate that with heating, the IgG-PBA of SLE sera did not increase to a greater degree than did that of normal sera.

In addition, the effect on serum IgG-PBA of adding increasing amounts of heat-aggregated normal human IgG to the unheated or heated normal serum samples was determined. The aggregates were equally divided between small (19S or less) and large ( $>19S$ ) sizes. The addition of  $\approx 25$   $\mu$ g heat-aggregated IgG/ml of fresh normal serum increased the value of IgG-PBA to that of heated serum (Fig. 2). Adding 25 to 500  $\mu$ g/ml of aggregated IgG to normal heated serum yielded levels of IgG-PBA equivalent to those seen in SLE sera (see below).

**IgG-PBA of serum.** The amount of IgG that bound to normal PMN after in vitro incubation with heat-in-

activated sera from 36 normal individuals, 6 patients with IgG monoclonal paraproteins, and 38 SLE patients was determined. The IgG-PBA (mean  $\pm$  1 SD) of the normal serum samples was  $29.2 \pm 8.3$  ng IgG/ $10^6$  PMN, as recently published (9). The mean value for the 6 monoclonal IgG sera, whose IgG concentrations varied from 1.4 to 5.8 gm/100 ml, was  $30.7 \pm 8.5$  ng IgG/ $10^6$  PMN, not significantly increased above normal serum IgG-PBA. (Five of the monoclonal IgG paraproteins were of subclass 1 or 3). On the other hand, the mean IgG-PBA for the SLE sera,  $53.6 \pm 21.3$  ng IgG/ $10^6$  PMN, was significantly greater than the mean values seen with sera from normals or patients with comparably elevated IgG levels ( $P < 0.001$ , Student's  $t$  test) (Fig. 3). Furthermore, 22 of the SLE sera (58%) had levels of IgG-PBA 2 SD or more above the normal mean. The levels of IgG-PBA seen with the SLE sera showed no correlation with the values for direct PMN-bound IgG on the patients' own cells ( $r = 0.27$ ,  $P > 0.20$ ). Also, the serum IgG concentrations and the blood neutrophil counts failed to correlate with the serum IgG-PBA levels in the patients.

**Serum fractionation studies.** To investigate whether the elevated levels of IgG-PBA of the SLE sera were caused by monomeric IgG antineutrophil antibodies or by IgG-containing immune complexes, 1.5 ml of 7 normal and 18 SLE sera were fractionated by Sephadex G-200 gel filtration. After concentrating each pool to  $\approx 1.5$  ml, a 200- $\mu$ l aliquot was incubated with normal PMN and the amount of cell-bound IgG was measured as for serum. Levels of IgG-PBA elevated

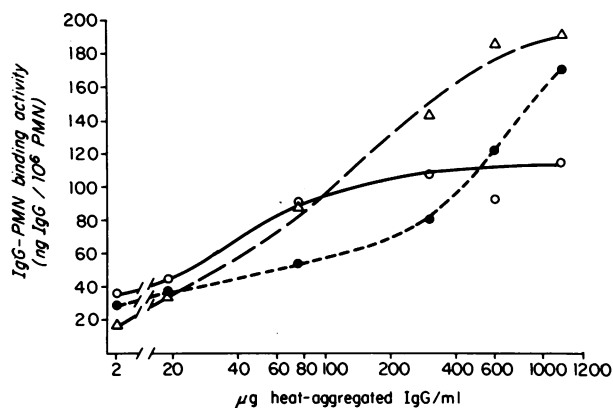


FIGURE 2 Effect on IgG-PBA of adding increasing amounts of heat-aggregated IgG to PBS ( $\Delta$ ), unheated normal serum ( $\bullet$ ), or heat-inactivated ( $56^\circ\text{C}$  for 30 min) normal serum ( $\circ$ ). The levels of IgG-PBA for serum and PBS without added aggregated IgG were the same as those obtained after the addition of 2  $\mu$ g/ml of aggregates. Aggregated IgG in the range of 100–1,200  $\mu$ g/ml caused a higher level of IgG-PBA when added to PBS, compared to normal serum. The values for IgG-PBA, shown on the ordinate, are on a linear scale; the amounts of IgG aggregates are plotted on the abscissa on a logarithmic scale.

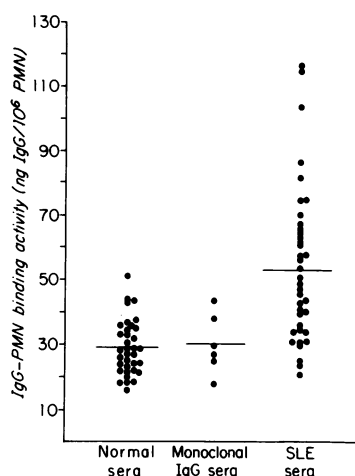


FIGURE 3 IgG-PBA in serum samples from 36 normal controls, 6 patients with monoclonal IgG paraproteins, and 38 patients with SLE. The mean value for the SLE sera was significantly increased over the mean observed with the normal or paraprotein-containing sera ( $P < 0.001$ , Student's  $t$  test).

more than 2 SD above normal were found in 11 G-200 excluded pools and in 13 of the 18 IgG pools; 9 sera contained elevated values in both pools (Table I).

No correlation was observed between the values for IgG-PBA and the IgG concentrations of the SLE G-200 IgG pools, which were between 250 and 1,000 mg/100 ml with a mean of 400 mg/100 ml. At this mean IgG concentration the IgG-PBA of the SLE G-200 IgG pools ranged from 26 to 90 ng IgG/ $10^6$  PMN. However, varying the protein concentration in an individual G-200 IgG pool did not affect the detected value of IgG-PBA, as shown in Fig. 4 for normal and two SLE samples. The differences in the values of IgG-PBA between these SLE and normal G-200 IgG pools remained fairly constant over the concentration range encompassing all of the SLE IgG pools, 250–1,000 mg/100 ml.

The level of IgG-PBA observed in each G-200 IgG pool of 18 SLE serum samples is plotted against the serum IgG-PBA in Fig. 5. The results indicate that a significant correlation existed between the levels of IgG-PBA in SLE sera and those in the G-200 IgG pools ( $r = 0.75$ ,  $P < 0.001$ ).

The levels of IgG-PBA in the SLE G-200 excluded pools also were not correlated with the total protein concentration in the pools. However, a similar effect of concentration on values of IgG-PBA was seen using serial dilutions of two SLE G-200 excluded pools. The values of IgG-PBA for the SLE G-200 excluded samples were plotted against the levels of serum IgG-PBA, and also demonstrated a positive correlation ( $r = 0.50$ ,  $P < 0.05$ ) (Fig. 6). The values for IgG-PBA in the albumin pools of normal or SLE sera were all in the same low range.

**SDG fractionation of SLE sera.** Four SLE sera with elevated levels of IgG-PBA were fractionated by SDG ultracentrifugation to determine the size distribution of the immune complexes. Increased values of IgG-PBA were present in serum fractions containing large immune complexes (19S or greater), intermediate complexes (7S–19S), or monomeric IgG (7S), for these four SLE sera (Table II). The elevated levels of IgG-PBA observed in the SLE albumin fractions represented spillover from the adjacent monomeric IgG peaks. To ascertain whether fractionating the serum led to an artifactual increase in the total IgG-PBA, the sucrose gradient fractions of one SLE serum were recombined and adjusted to the original serum IgG concentration. The level of IgG-PBA in the recombined pool was only 18% greater than the value observed with the original serum sample. These data demonstrated that IgG-containing immune complexes of diverse sizes, as well as monomeric IgG antineutrophil antibodies, contributed to the elevated levels of IgG-PBA in SLE sera and that the pattern of activity varied between individual serum samples.

TABLE I  
IgG-PBA of G-200 Pools of Normal and SLE Sera

	Whole serum	G-200 pools	
		Excluded	IgG
	ng IgG/ $10^6$ PMN		
Normal*	29.2±8.3 (n = 36)	54.71±13.2 (n = 7)	34.1±7.7 (n = 7)
SLE patients†			
J.B.	88.2	153.6	77.7
H.L.	45.2	110.4	90.8
B.C.	64.7	115.4	68.6
K.T.	72.7	110.1	98.1
M.S.	37.4	39.3	58.5
S.M.	41.3	85.4	50.1
D.H.	81.5	68.0	87.0
C.F.	48.4	126.9	46.1
J.U.	52.9	211.5	52.7
D.Z.	20.1	54.9	35.3
N.L.	55.9	140.4	87.3
P.A.	70.6	207.7	88.5
S.W.	41.0	98.5	49.8
L.A.	39.1	98.0	39.2
L.L.	37.9	52.0	59.4
C.C.	44.7	59.0	37.8
B.R.	54.0	54.9	51.8
F.D.	25.9	54.2	26.6

\* Results obtained from 36 normal sera or from the excluded and IgG pools from 7 normal sera. The data are expressed as the mean±1 SD.

† The results from 18 SLE patients are expressed as the mean based upon two or more data points in each instance.

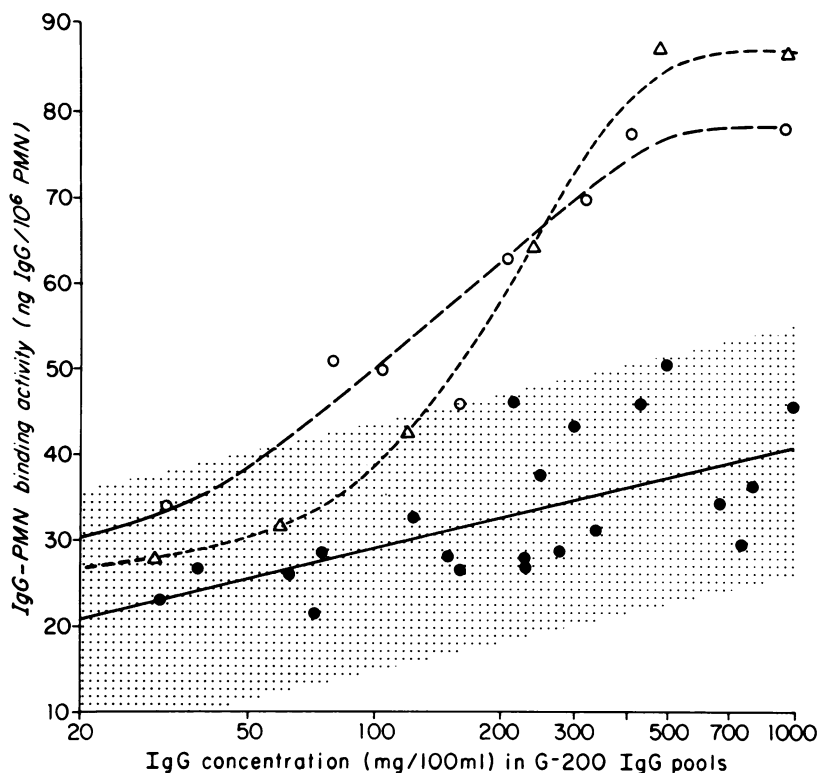


FIGURE 4 Effect of IgG concentration on the IgG-PBA of Sephadex G-200 included (IgG) pools from normal sera (●), and two SLE patients, J.B. (○) and D.H. (△). The IgG concentration, shown on the abscissa, is on a logarithmic scale. The shaded area represents 2 SD above and below the normal mean.

**Immunoabsorption of anti-F(ab')<sub>2</sub>.** To exclude the possibility that the elevated levels of IgG-PBA detected in the G-200 excluded pools of the SLE sera were caused by IgM antineutrophil antibodies, the rabbit antihuman F(ab')<sub>2</sub> antiserum was rendered specific for the Fd portion of human IgG as outlined in Methods. Compared to the unadsorbed antiserum, however, 30% more normal human IgG was required to produce equal inhibition of binding of the <sup>125</sup>I-Fab by the adsorbed anti-F(ab')<sub>2</sub> antiserum. Thus, the absolute values of IgG-PBA detected with the adsorbed antiserum were 30% less than those detected with the unadsorbed antiserum. The IgG-PBA of 17 of the SLE G-200 excluded pools were determined with the adsorbed antiserum and correlated well with those obtained with the unadsorbed antiserum ( $r = 0.79$ ,  $P < 0.001$ ). The mean IgG-PBA of the SLE G-200 excluded pools was 100.3 ng IgG/10<sup>6</sup> PMN with the unadsorbed antiserum, compared to a mean of 65.0 with the Fd $\gamma$ -specific antiserum. The absolute amounts of IgG-PBA detected in the G-200 excluded pools, therefore, were identical with the two antisera when corrections were made for the 30% shift in the standard inhibition curve noted above. Furthermore, the same SLE G-200 excluded pools exhibited levels of IgG-PBA that were elevated

2 SD or more above the normal mean when using the absorbed or original antisera. Approximately 10% of the total protein in the G-200 excluded pools of normal or SLE sera was IgG, as determined by radial immunodiffusion. These results indicated that the elevated levels of IgG-PBA present in the G-200 excluded pools of SLE patients were not a result of IgM antineutrophil antibodies but represented IgG-containing immune complexes.

**Pepsin digestion of IgG.** These studies were carried out to confirm that the monomeric IgG antibodies from SLE sera attached via their antibody-combining sites to the neutrophils. The F(ab')<sub>2</sub> fragments of G-200 purified IgG were prepared from five normal and seven SLE sera with elevated values of IgG-PBA. The amount of binding to normal PMN was determined as outlined in the Methods. In contrast to IgG, the amount of binding to PMN by F(ab')<sub>2</sub> from normal IgG increased to  $92.5 \pm 24.1$  ng F(ab')<sub>2</sub>/10<sup>6</sup> PMN (mean  $\pm$  1 SD). Nevertheless, the level of PMN binding observed with the F(ab')<sub>2</sub> from the SLE sera,  $153.5 \pm 44.6$  ng F(ab')<sub>2</sub>/10<sup>6</sup> PMN, was significantly greater than that seen with normal F(ab')<sub>2</sub> ( $P = 0.02$ , Student's  $t$  test) (Table III). Increased binding to normal PMN was observed with six of the seven SLE F(ab')<sub>2</sub> preparations. Similar

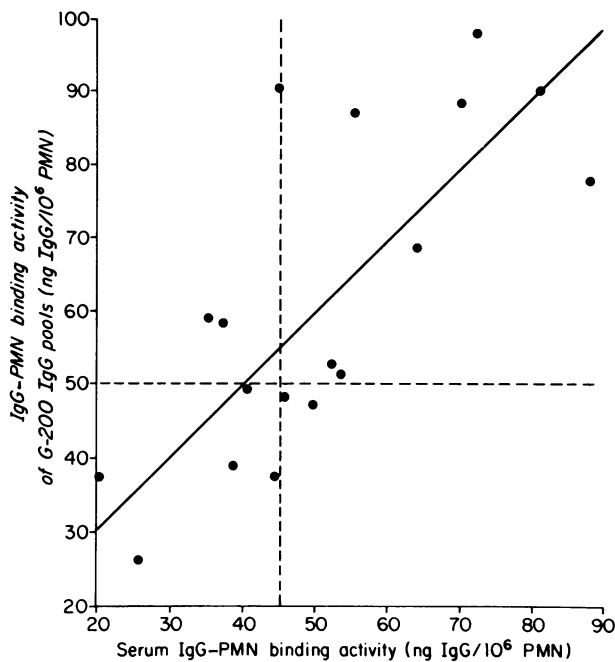


FIGURE 5 Levels of IgG-PBA in G-200 included (IgG) pools from 18 SLE patients plotted against the values of IgG-PBA for whole sera. The two values showed a significant correlation ( $r = 0.75$ ,  $P < 0.001$ ). Values 2 SD above the mean are shown for IgG-PBA of normal sera (vertical line ---) and of G-200 included pools at an IgG concentration of 400 mg/100 ml (horizontal line ---) from normal sera.

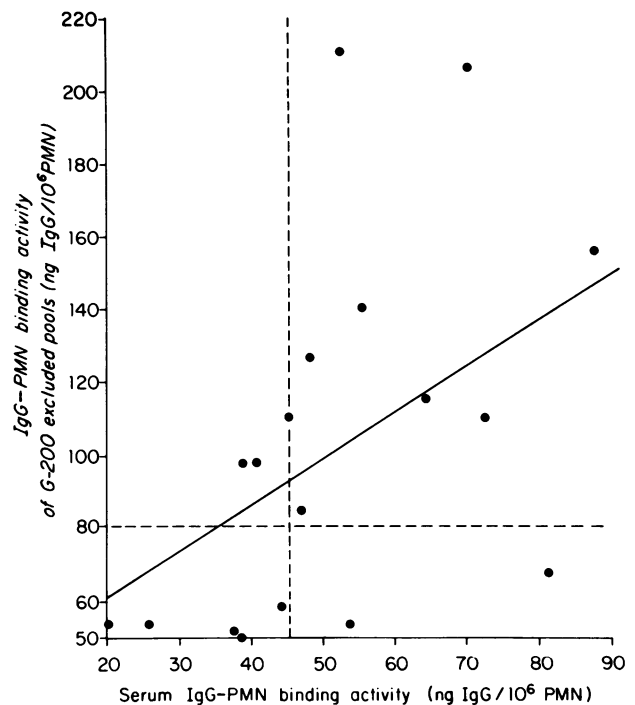


FIGURE 6 Levels of IgG-PBA in 18 SLE G-200 excluded pools plotted against the values of IgG-PBA in the SLE sera ( $r = 0.50$ ,  $P < 0.05$ ). Values 2 SD above the mean IgG-PBA for normal sera or G-200 excluded pools are shown as described in the legend for Fig. 5.

results were observed with normal and SLE F(ab')<sub>2</sub> obtained from DEAE-purified IgG after removal of pepsin agglutinators. These data indicated that IgG antibodies to neutrophils contributed to the elevated IgG-PBA seen with some SLE sera.

*Immunofluorescence of PMN-bound IgG.* These studies were performed to determine whether the elevated IgG-PBA seen with SLE sera could be a result of ANA binding to nuclei of PMN damaged during purification. Seven normal and nine SLE sera with elevated

TABLE II  
IgG-PBA of SDG Fractions of Normal and SLE Sera

		SDG fractions				
	Whole serum	I (>19S)	II (19S)	III (7S-19S)	IV (7S)	V (<7S)
ng IgG/10 <sup>6</sup> PMN						
Normal*	29.2±8.3	62.5±11.4	48.0±15.6	41.3±9.6	32.7±10.6	14.9±2.0
SLE†						
B.C.	116.7±31.0	114.2±20.2	86.1±22.4	89.6±25.6	62.5±4.8	20.5±3.9
K.T.	98.7±5.3	82.4±6.8	94.3±51.2	63.3±10.1	56.9±17.6	22.1±3.5
D.H.	70.3±15.2	70.6±10.7§	88.1±19.9	63.5±10.6	103.0±36.4	27.7±4.0
J.B.	81.4±5.3	119.5±32.0	98.9±38.0	77.1±19.6	51.7±3.8	35.2±3.4

\* Results obtained from sera and fractions from four normal individuals. The data are expressed as mean±1 SD based upon at least 30 total values from each gradient fraction obtained in nine separate experiments.

† Results obtained from four SLE patients expressed as mean±1 SD based upon at least four values from two separate experiments for each SLE serum sample and gradient fraction. All mean values are significantly different from normal mean values ( $P < 0.001$ , Student's *t* test) except as noted.

§  $P > 0.05$  (Student's *t* test), not significantly different from normal value.

TABLE III  
PMN Binding of F(ab')<sub>2</sub> from Normal and SLE Sera

SLE patient*	PMN-bound F(ab') <sub>2</sub> †	Normal	PMN-bound F(ab') <sub>2</sub> †
ng F(ab') <sub>2</sub> /10 <sup>6</sup> PMN			
K.T.	188.7	K.K.	93.6
M.S.	122.9	J.S.	132.0
K.M.	163.8	J.V.	72.1
M.M.	227.6	M.B.	74.3
J.B.	95.2	D.S.	90.5
B.C.	126.6		
D.H.	150.0		
mean ± 1 SD	153.5 ± 44.6§		92.5 ± 24.1

\* All patients had elevated values of IgG-PBA of sera and G-200 IgG pools.

† F(ab')<sub>2</sub> obtained by pepsin digestion of G-200 IgG pools was incubated with normal PMN as outlined in Methods. Results are expressed as the mean from at least two data points.

§ Significantly greater than mean value of PMN binding by F(ab')<sub>2</sub> from normal sera ( $P = 0.02$ , Student's  $t$  test).

IgG-PBA were first incubated with normal PMN in the usual manner. After six washes, these cells were then stained with fluorescein isothiocyanate-labeled F(ab')<sub>2</sub> fragments of goat antihuman F(ab')<sub>2</sub> as outlined in Methods. Neutrophils incubated with SLE sera exhibited a bright speckled pattern on the surface of virtually all cells (Fig. 7). Similarly, PMN incubated with G-200 IgG pools from the SLE sera had positive surface immunofluorescence on all cells. Neutrophils incubated with normal sera or with PBS exhibited only trace immunofluorescence.

**Other studies on SLE sera.** 22 SLE sera were examined for the presence of immune complexes using the Raji assay. 15 of these sera were positive, exhibiting >12 µg aggregated human IgG equivalents/ml serum. The IgG-PBA was elevated in 16 out of these 22 SLE sera. The absolute levels of serum IgG-PBA were strongly correlated with the results obtained with the Raji assay ( $r = 0.74$ ,  $P < 0.001$ ).

The ANA was positive in 21 of these 22 SLE sera. A significant correlation was seen between the ANA titers and the levels of IgG-PBA ( $r = 0.68$ ,  $P < 0.002$ , Spearman's rank correlation method) as well as with the results obtained with the Raji assay ( $r = 0.61$ ,  $P < 0.005$ , Spearman's rank correlation method).

Elevated levels of serum PMN-opsonizing ability were seen in only 3 of these 22 SLE sera. Each of these three sera had very high levels of IgG-PBA in whole serum, as well as in both G-200 excluded and IgG pools. These three sera also exhibited the highest ANA titers and the most abnormal values in the Raji assay. Two of these three patients had elevated levels of IgG bound to their own PMN. The absolute PMN count, however,

was normal in these three SLE patients with elevated levels of serum PMN-opsonizing ability.

## DISCUSSION

Increased amounts of IgG on or in PMN from most SLE patients have been found by other investigators using qualitative or semiquantitative techniques (1, 2). The objectives of the present experiments were to quantify this PMN-bound IgG and to determine the contributions of soluble immune complexes and anti-PMN antibodies in SLE sera to the levels of IgG-PBA. Since PMN have receptors for the Fc portion of IgG (22), the accurate determination of abnormal amounts of IgG on these cells presents technical difficulties. Both cell-

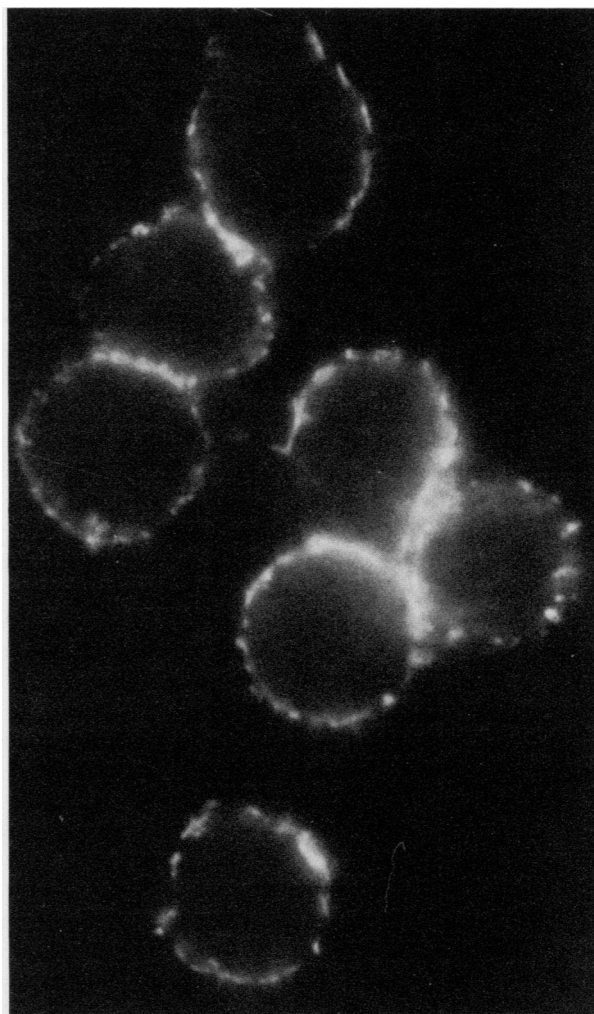


FIGURE 7 Immunofluorescence of normal neutrophils incubated with serum from a patient (J.B.) with SLE. After six washes, the cells were stained with fluorescein isothiocyanate-labeled F(ab')<sub>2</sub> fragments of goat antihuman F(ab')<sub>2</sub>. Neutrophils incubated with normal serum gave only trace immunofluorescence. Original magnification ×1,000.



surface and interiorized IgG will be detected by the assay for direct PMN-bound IgG; thus, the results may reflect some IgG interiorized in vivo or during the cell separation procedures. The values for serum IgG-PBA, however, reflect only membrane-bound IgG and not aggregates or complexes possibly engulfed during in vitro incubation of sera with the normal cells as the latter were preincubated with 2-deoxyglucose to block interiorization (10).

The increase in IgG-PBA induced by heating sera to inactivate complement was shown to be no greater with SLE than with normal sera. Also, normal values of IgG-PBA were obtained with heated sera containing IgG paraproteins, including those of subclasses 1 and 3 in concentrations equal to or greater than the IgG in SLE sera. Furthermore, the IgG concentrations in the SLE sera showed no relationship to the levels of IgG-PBA. The elevated values for IgG-PBA found with SLE sera were therefore concluded to be caused by soluble complexes or to antibodies directed against granulocytes, and not to nonspecific adherence of IgG to the cells.

The results of our studies showed elevated values for IgG-PBA in G-200 excluded pools of SLE sera and in sucrose gradient fractions containing large- (19S or greater) or intermediate- (7S–19S) sized immune complexes as well as in monomeric IgG fractions. The sum of the values for IgG-PBA in the separate SDG fractions from normal or SLE sera exceeded the levels observed with the respective whole sera. Yet the level of IgG-PBA in the recombined fractions of one SLE serum was only minimally elevated over the value obtained with the original whole serum. Fractionation of these sera may have led to the separation of materials, such as monomeric IgG, capable of inhibiting the binding of the complexes or aggregates to the cells in a nonspecific fashion (23, 24). In addition, IgG anti-PMN antibodies, or other unidentified materials, may have specifically blocked the Fc receptors on the cells. Assays performed with an Fd $\gamma$ -specific antiserum confirmed that the cell-binding activity in the high molecular weight pools was caused by IgG-containing complexes and not by IgM anticell antibodies. Additional evidence for binding of immune complexes in these SLE sera to PMN was the high degree of correlation noted between the values of IgG-PBA and the results of the Raji assay for immune complexes.

The levels of IgG-PBA observed with G-200 IgG pools from normal sera may represent nonspecific adherence or the presence of low levels of anti-PMN antibodies; leukocyte-reactive antibodies have been detected in low titers in normal sera using cytotoxicity assays (25, 26). The elevated levels of IgG-PBA found in the G-200 IgG pools of SLE sera were concluded to be caused by the presence of IgG antibodies reactive with neutrophils. The finding of greater PMN-

binding by F(ab') $_2$  from SLE sera than F(ab') $_2$  from normal sera offers further support for this conclusion. The high degree of correlation between the values of IgG-PBA in the SLE sera and those in the monomeric IgG serum fractions suggests that antineutrophil antibodies may contribute significantly to the elevated levels of IgG-PBA in sera, and possibly to the increased amounts of IgG found on SLE PMN in vivo. Other investigators have described increased levels of cold-reactive granulocytotoxic antibodies of undetermined immunoglobulin class in over 50% of 57 SLE sera (26).

The nature of the stimulus for producing antibodies that bind to neutrophils in SLE is unknown. Their ubiquity suggests that they may result from nonspecific B-cell hyperreactivity (27, 28). As postulated by Steinberg and Reinertsen (29), autoantibody-producing B cells in SLE might encounter antigens in the bone marrow such as those on cells involved in hematopoiesis and granulopoiesis, leading to production of autoantibodies to erythrocyte, leukocyte, or nuclear antigens.

The nature of the antigen(s) recognized by antibodies to neutrophils in SLE has not been established. The results of our immunofluorescence studies demonstrate that the antigen is present on the neutrophil surface and is not associated with the nucleus. It is unlikely that allo-antibodies represent the principle activity, since the IgG-PBA of the G-200 IgG pools from at least three SLE sera gave comparably elevated levels when tested with four or more neutrophil donors (unpublished observation). Recent studies have detected antibodies to beta-2-microglobulin in  $\approx 20\%$  of SLE sera (30). Since this protein is present on the surface of neutrophils as a subunit of HLA antigens (31), such antibodies could bind to these cells. In other studies, ANA that cross-react with lymphocyte and granulocyte surface antigens have been detected in 5 out of 24 ANA-positive sera (32). Only one of the five sera was from an SLE patient. Significant correlations were observed in our studies between the levels of serum IgG-PBA and the ANA titers; however, we did not examine for any possible cross-reactivity. Thus, to determine whether these antibodies recognize antigens specific to neutrophils or antigens associated with neutrophils, as well as other cells, will require further studies.

Soluble immune complexes or anticell antibodies could influence PMN function or kinetics in SLE patients. We have previously reported extensive studies on an SLE patient with severe granulocytopenia, offering evidence that IgG antibodies to neutrophils mediated destruction of his cells through an autoimmune mechanism (9). In other studies, anti-PMN antibodies injected into rabbits resulted in rapid cell depletion by opsonizing neutrophils for ingestion by fixed tissue macrophages in the liver and spleen (33). Homologous anti-PMN antibodies studied in vitro inhibited the ingestion of opsonized particles and coated

the neutrophils for ingestion by other phagocytic cells (34). The infrequent detection of PMN-opsonizing antibodies in our SLE sera may reflect a lower sensitivity of this assay or a failure of the SLE antibodies to opsonize because of a sparse distribution of antigens on the cell surface.

It should be emphasized that the majority of SLE patients (35), including ours, are not neutropenic. The lack of correlation between the levels of neutrophil-bound IgG or serum IgG-PBA and the PMN counts in our patients appears paradoxical. These observations suggest the possible role of other factors, such as antibody-binding characteristics, antigen site density, or phagocytic cell function. Also, neutrophil kinetic studies were not performed on any of these SLE patients. Antibodies or immune complexes that bind to platelets and decreased platelet survival or increased blood megathrombocytes were detected in 75% of SLE patients (36). The finding of thrombocytopenia in only 14% of SLE patients, however, suggested that a compensated thrombolytic state was common in SLE (36). Further neutrophil kinetic studies in SLE patients will be necessary to establish whether a similar situation of increased peripheral destruction and compensated bone marrow production exists for this cell as well.

The interaction of complement with neutrophils also may affect their function or kinetics (37, 38). Activated complement components have recently been described on circulating PMN from SLE patients (39). The results of our studies suggest that complement could be activated on the neutrophil surface in SLE patients either through adherent immune complexes or by anti-PMN antibodies.

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