

## **Studies with human leukocyte lysosomes. Evidence for antilyosome antibodies in lupus erythematosus and for the presence of lysosomal antigen in inflammatory diseases.**

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### **Research Article**

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# Studies with Human Leukocyte Lysosomes

## EVIDENCE FOR ANTILYSOSOME ANTIBODIES IN LUPUS ERYTHEMATOSUS AND FOR THE PRESENCE OF LYSOSOMAL ANTIGEN IN INFLAMMATORY DISEASES

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**ABSTRACT** Human lysosomes were isolated from normal peripheral blood leukocytes and characterized by electron microscopy, enzyme analysis, and assays for DNA and RNA. Stored sera from 37 unselected patients with systemic lupus erythematosus (SLE), including active and inactive, treated and untreated cases, were tested in complement fixation (CF) reactions with these lysosome preparations. 23 SLE sera exhibited positive CF reactions, as did sera from two patients with "lupoid" hepatitis. The seven SLE sera with strongest CF reactivity also demonstrated gel precipitin reactions with lysosomes. Neither CF nor precipitin reactions with lysosomes were observed with normal sera or with sera of patients with drug-induced lupus syndrome, rheumatoid arthritis (RA), polymyositis, or autoimmune hemolytic anemia. By several criteria the antilyosome CF and precipitin reactions of SLE sera could not be attributed to antibody to DNA, RNA, or other intracellular organelles. The lysosomal component reactive with SLE sera in CF assays was sedimentable at high speed and is presumably membrane associated. The CF activity of two representative SLE sera was associated with IgG globulins by Sephadex filtration.

This work was presented in part at the 35th Annual Meeting of The Arthritis and Rheumatism Association (1). Dr. Bell was a Postdoctoral Fellow of the Medical Research Council of Canada and his present address is University Hospital, University of Western Ontario, London, Ontario, Canada. Dr. Leddy was a Senior Investigator of the Arthritis Foundation. A portion of this work was in partial fulfillment of the requirements for a Master's degree in Microbiology by Miss Thiem.

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A search for lysosomal *antigen* in SLE and related disorders was also made. By employing rabbit antiserum to human lysosomes in immunodiffusion, a soluble lysosomal component, apparently distinct from the sedimentable (membrane-associated) antigen described above, was identified in serum, synovial fluid, or pleural fluid from patients with SLE, RA, ankylosing spondylitis, and leukemoid reaction. An antigenically identical soluble component reactive with the rabbit antiserum could be released in vitro from intact lysosomes by repeated freeze-thaw cycles. Thus, the naturally occurring soluble antigen may be liberated from leukocytes in vivo as a normal aspect of inflammation, and clearly is not unique to SLE. In those SLE patients possessing antilyosome antibodies, however, release of lysosomes at sites of inflammation could lead to local formation of immune complexes with potential for perpetuating tissue injury.

### INTRODUCTION

The sera of patients with systemic lupus erythematosus (SLE)<sup>1</sup> characteristically contain antibodies reactive with a variety of plasma, cellular, and subcellular constituents. Among these, autoantibodies to nuclear antigens have been the most extensively studied, and DNA: anti-DNA complexes have been implicated in the development of the renal and vascular lesions of this

<sup>1</sup> *Abbreviations used in this paper:* ANA, antinuclear antibody; CF, complement fixation; EA, sensitized sheep red cells; E:S, enzyme:substrate; RA, rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; SN, supernate; VBS, Veronal-buffered saline.

disease (2-6). Antibodies to several types of cytoplasmic antigens have also been described (7-15) but their pathogenetic importance in SLE is less clear.

Previous studies on the occurrence of antibodies to lysosomes in SLE were based upon complement fixation (CF) reactions with relatively crude cytoplasmic fractions derived to a large extent from nonhuman tissues (10, 16). The present investigation was designed to reexamine the question of antibodies to lysosomes in SLE, with particular respect to: (a) immune reactivity of SLE sera with extensively characterized preparations of human lysosomes by both CF and immunodiffusion techniques; and (b) the possible presence of lysosomal antigen(s) in the sera or pathologic fluids of SLE patients. For comparison, similar studies were also undertaken in patients with other rheumatic or immunologic disorders.

## METHODS

### Preparation and characterization of antigens

*Lysosome granules* were prepared from the peripheral blood leukocytes of several normal group O donors by a slight modification of the method of Chodirker, Bock, and Vaughan (17). 100 ml of freshly drawn venous blood was immediately diluted in 1,900 ml of cold isotonic saline containing 0.1% bovine serum albumin and subjected to cold centrifugation in 250-ml glass centrifuge bottles (800 *g*, 30 min) to recover the cells. After three cycles of hypotonic saline lysis of red cells, the remaining leukocytes were recovered by cold centrifugation (110 *g*, 5 min) and then washed successively in cold isotonic saline and 0.34 M sucrose. The washed leukocytes were lysed in 0.2 M sucrose containing 3.6 mg (500 U) per ml of heparin (17). Overnight cold incubation (4°C) of leukocyte lysates was followed by low-speed centrifugation (800 *g*, 15 min) to remove cell debris and this was followed by centrifugation (25,000 *g*, 10 min) at 4°C to sediment the lysosome granules. The granules were resuspended in 0.34 M sucrose (containing 250 U of heparin per ml), washed in heparin-free 0.34 M sucrose, then cleared of lysosomal clumps (centrifugation at 600 *g*, 10 min) before use. All centrifugations were carried out in refrigerated centrifuges (PR 2, International Equipment Co., Needham Heights, Mass.; RC 2B, Ivan Sorvall, Inc., Newtown, Conn.; L-240, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

The final yield of lysosome granules in such preparations was 3-4 ml of a suspension containing approximately 1 mg protein/ml by Folin analysis using an egg albumin standard (18). The preparations were promptly examined by phase-contrast microscopy (1,000 $\times$ ) and typically showed a myriad of granules dancing freely in Brownian movement. Granule preparations were discarded if they contained recognizable red cells, leukocytes or platelets, or more than occasional cell debris or granule clumps. As a further test of lysosomal purity, electron micrographs of two representative preparations were kindly prepared by Dr. Goetz W. Richter, Department of Pathology, University of Rochester. Lysosomal granules were sedimented (25,000 *g*, 10 min), fixed with 2% glutaraldehyde (3 h) and 1%  $O_3O_4$  (1 h), and stained with uranyl acetate (20 min) and lead citrate (5 min). These electron micrographs (Fig. 1) revealed that virtually all of the particles

possessed typical morphologic features of lysosomes. Recognizable cells were not observed. Occasional fragments of cell membrane or of other subcellular organelles were found in some fields.

Acid phosphatase, myeloperoxidase, and neutral protease activities were readily detectable in fresh preparations of lysosomes but were greatly increased by disruption of the granules with 0.1% Triton X-100. Acid phosphatase was measured as described by Janoff, Weissman, Zweifach, and Thomas (19) with *p*-nitrophenyl phosphate as substrate. Myeloperoxidase was assayed with *O*-dianisidine as substrate, according to Klebanoff (20). Neutral protease activity was assayed by digestion of denatured hemoglobin (21, 22) but in a pH 7.3 phosphate-buffered saline system instead of acid buffer.  $\beta$ -Glucuronidase was also measured in such lysosome preparations when the method of isolation was being developed (17). Succinic dehydrogenase (23) and reduced NAD dehydrogenase (cytochrome *c* reductase) (24) activities were not detected in the current lysosome preparations.

( $Na^+$ ,  $K^+$ )-ATPase activity was measured in lysosome membranes as a test for contamination by fragments of leukocyte plasma membrane (25). This assay, kindly performed by Dr. F. H. Kirkpatrick, Department of Radiation Biology and Biophysics (Rochester), is based on cleavage of [ $\gamma$ - $^{32}P$ ]ATP in a sensitive modification\* of the method of Berenblum and Chain (26). ATPase activity was measured in the presence of 1 mM Tris ATP (pH 7.2), 1.5 mM  $MgCl_2$ , 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) buffer (pH 7.2), and either 65 mM NaCl or 50 mM NaCl plus 15 mM KCl. Inhibition was tested with  $10^{-4}$  M ouabain. For this procedure lysosomes from two preparations (672 and 400  $\mu$ g protein, respectively) were subjected to 10 cycles of freezing and thawing followed by sedimentation of the membranes at 31,000 *g* for 30 min and three washes in 20 vol 0.34 M sucrose. Background ATPase was readily demonstrated in both preparations of lysosome membranes in the presence of  $Mg^{++}$  ( $240 \pm 8$  and  $180 \pm 8$  nmol/mg protein/h, respectively). Significant ( $Na^+$ ,  $K^+$ )-dependent ATPase activity was not detected ( $< 17 \pm 8$  and  $< 16 \pm 12$  nmol/mg protein/h, respectively), either as stimulation of  $Mg^{++}$ -ATPase by  $Na^+ + K^+$ , or as inhibition by ouabain of  $Mg^{++}$ -dependent or ( $Na^+$ ,  $K^+$ )-stimulated activity.

Lysosomes were also obtained by an alternative protocol obtained from Dr. Robert I. Lehrer, University of California. Briefly, heparinized blood was subjected to (a) low-speed centrifugation to remove platelets and plasma; (b) centrifugation at 400 *g* in a Ficoll-34% Hypaque gradient, yielding a mononuclear layer (discarded) and a granulocyte-red cell pellet; (c) sedimentation of the latter fraction in 3% dextran followed by hypotonic lysis to remove red cells; (d) resuspension of granulocytes in 0.34 M sucrose and homogenization in a glass-Teflon homogenizer; (e) washes in 0.34 M sucrose to remove debris and final recovery of lysosomes by centrifugation in cellulose nitrate tubes in a Spinco model L ultracentrifuge (SW-50 rotor) for 20 min at 27,000 rpm. The yield of lysosomes in our hands was comparable to that in our standard procedure. The second method was selected because it avoids exposure of subcellular structures to high concentrations of heparin, which has been shown to release DNA and RNA from rat liver nuclei (27).

\* Kirkpatrick, F. H., G. M. Woods, and P. L. LaCelle. 1974. Multiple ATPase activities of spectrin: stimulation by calcium and magnesium. Submitted for publication.

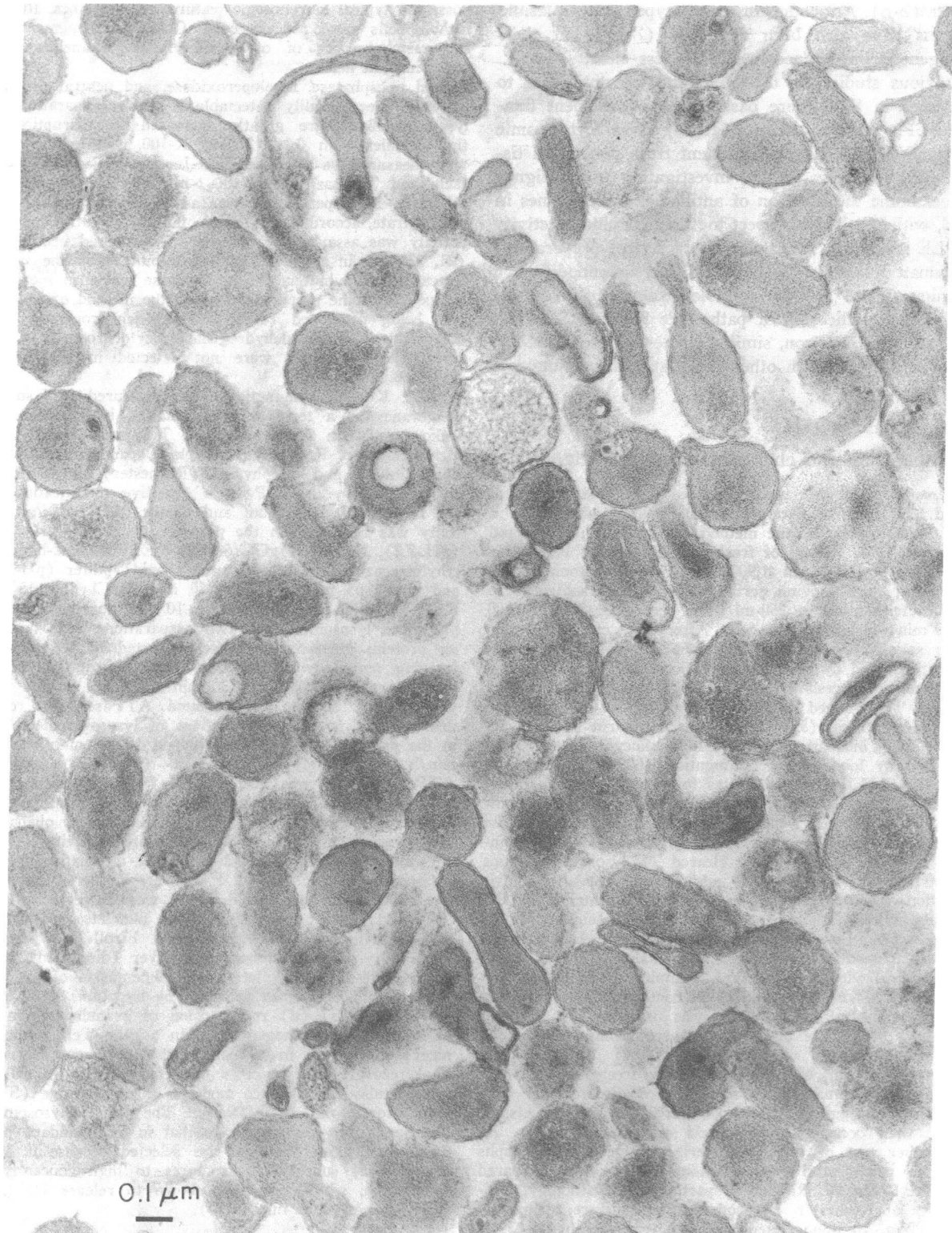


FIGURE 1 Electron micrograph of lysosome granules isolated from human peripheral blood leukocytes.  $\times 58,000$ .

Assay of lysosome preparations for DNA was performed by the diphenylamine method with calf thymus DNA (Worthington Biochemical Corp., Freehold, N. J.) as standard (28). RNA was kindly assayed by Dr. Arnold Meisler, Departments of Medicine and Microbiology (Rochester), using the method of Scott, Fraccastoro, and Taft (29). Soluble nucleoprotein and Sm antigens were prepared in the laboratory of Dr. Eng Tan, Scripps Clinic and Research Foundation, as previously described (30, 31). Crystalline pancreatic DNase (2,460 U/mg) and RNase (3,095 U/mg) were purchased from Worthington Biochemical Corp. [ $^3\text{H}$ ]DNA from *Escherichia coli*, extracted by modifications of the procedure of Lehman (32), was supplied by Dr. Robert Swift, Department of Microbiology (Rochester). 28S [ $^{14}\text{C}$ ]RNA, phenol purified from rat liver, was the gift of Dr. Meisler.

Experiments were undertaken to determine whether treatment of lysosomes with high concentrations of DNase or RNase would affect their capacity to give CF reactions (series I) or precipitin reactions (series II) with SLE sera. For this purpose the lysosomes were assumed to have DNA or RNA contamination equivalent to one-tenth the lysosomal protein. In series I, DNase was employed in an enzyme:substrate (E:S) ratio of 1:2 in the presence of 0.01 M  $\text{MgCl}_2$  and incubation with lysosomes was at 37°C for 60 min; RNase was employed in an E:S ratio at 2:1 at 5°C for 60 min with gentle mixing. In series I concurrent control tubes containing added [ $^3\text{H}$ ]DNA or [ $^{14}\text{C}$ ]RNA, in addition to lysosomes and the appropriate nuclease (or diluent), permitted demonstration of effective cleavage of DNA or RNA under the conditions of the experiment (see Results). For this purpose the remaining radioactivity precipitable in 10% TCA was trapped on glass fiber filters and counted in a liquid scintillation counter, according to the general method of Meisler and Tropp (33). In series II, treatment of lysosomes with either DNase or RNase was performed at 37°C at an E:S ratio of 1:2 (for 60 min) or 1:10 (for 18 h), with 0.15 M  $\text{MgCl}_2$  provided in the DNase studies.

### Detection of antilyosome antibodies

**Quantitative CF tests** were patterned after the method of Wassermann and Levine (34). Sensitized sheep red cells (EA),  $5 \times 10^7$  cells/ml, in isotonic Veronal-buffered saline (VBS) containing  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  were prepared as previously described (35). Only freshly prepared lysosomes, stored no more than 3 days at 5°C, were used for these assays. Into 10-ml tubes in an ice bath were placed 0.5 ml diluent (see below), 0.25 ml diluted test serum, 0.25 ml reconstituted lyophilized guinea pig serum containing 10  $\text{CH}_{50}$  U/ml, and, lastly, 0.5 ml lysosomes diluted in 0.34 M sucrose, typically to 5 or 25  $\mu\text{g}/\text{ml}$ . Antigen (lysosome), antibody (serum), and complement control tubes were run in duplicate in each experiment. The tubes were incubated at 4°C for 18–20 h, then chilled in ice, and 0.25 ml of EA were added to each tube. Incubation then proceeded at 37°C until the complement controls indicated 100% lysis. The tubes were centrifuged and the  $\text{OD}_{412\text{ nm}}$  of each supernate was measured spectrophotometrically. The final percent of CF was calculated after subtracting the sum of any complement consumption occurring in the antigen (lysosome) control and serum control. In practice this total correction seldom exceeded 4–5%, owing to the pretreatment described below. The CF values shown in the tables have all been corrected for these anticomplementary effects. Although normal sera produced no detectable CF, SLE sera which

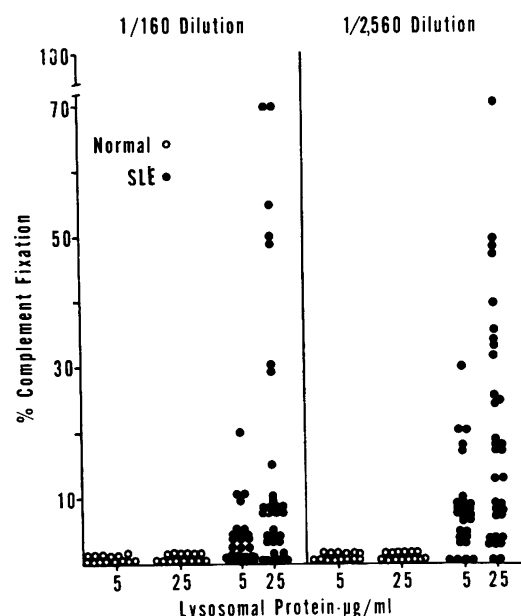


FIGURE 2 Histogram showing range of CF reactions of SLE and normal sera against human leukocyte lysosomes at concentrations of 5 and 25  $\mu\text{g}$  protein/ml. Weaker reactions obtained with lysosomes at 1  $\mu\text{g}/\text{ml}$  and 125  $\mu\text{g}/\text{ml}$  are not shown.

failed to give at least 10% corrected CF at any dilution were listed as negative in Table I. With each CF assay of unknown sera one or more known positive SLE sera and the rabbit antilyosome serum were included as positive controls.

Successful development of the above CF system depended upon overcoming substantial anticomplementary activity of the lysosomes themselves and of many SLE sera. The anticomplementary effect of lysosomes was circumvented by incorporating additional "carrier" protein in the system, i.e., the diluent was VBS containing 1:12 heat-inactivated normal human serum. Before CF assays, SLE sera were pretreated as follows: 3 ml 1:10 serum (in VBS) was incubated with 70  $\text{CH}_{50}$  U of guinea pig complement at 37°C for 60 min and then inactivated at 56°C for 30 min. This procedure<sup>a</sup> markedly reduced anticomplementary activity of SLE sera but did not hinder positive CF reactions with lysosomes. For uniformity, all human sera to be tested, including normals, were pretreated in this manner.

Fractionation of 1-ml aliquots of selected SLE sera was performed at 5°C in a 2.5 × 90-cm Sephadex G-200 column in borate-saline buffer, pH 8.0, with a flow rate of 8 ml/h. IgG-, IgA-, IgM-, and albumin-rich fractions were determined by immunodiffusion against specific antisera. Representative fractions were tested in CF assays against lysosomes (see below).

**Precipitin analysis in gel.** Tests for precipitating antibody against various antigens were made by double diffusion in glass petri dishes containing 25 ml freshly prepared 0.4% agarose gel in phosphate-buffered saline, pH 7.3, producing a gel thickness of 5 mm. Wells were 8 mm in diameter and placed 4 mm apart. When intact lysosomes

<sup>a</sup> Kindly suggested by Dr. Peter H. Schur, Robert Breck Brigham Hospital, Boston, Mass.

TABLE I  
*CF and Precipitin Reactions of SLE Sera with Human Leukocyte Lysosomes\**

Patient	Percent CF‡						Gel precipitin reaction
	40	160	Serum dilution (reciprocal)		10,240	40,960	
			640	2,560			
			%				
1. Tay.	31	53	30	47	21	4	+
2. Tho.	78	63	NT§	NT	27	0	+
3. Mor.	68	53	50	NT	NT	NT	+
4. Mem.	21	28	25	31	15	4	+
5. Kli.	8	29	24	31	12	1	+
6. Mai.	38	42	39	38	0	NT	+
7. Mend.	22	48	52	7	NT	NT	+
8. Aik.	14	14	19	26	6	2	0
9. Cre.	8	17	23	14	NT	NT	0
10. Del.	1	4	12	23	8	0	NT
11. Cia.	8	10	10	9	NT	NT	0
12. Wel.	3	6	8	13	1	0	0
13. Dic.	5	4	7	12	3	2	0
14. Bob.	2	5	10	13	1	NT	0
15. Ciac.	0	5	9	15	8	3	NT
16. Fal.	0	0	8	16	4	3	NT
17. Mur.	1	8	13	17	7	0	NT
18. Cer.	1	10	12	14	8	4	NT
19. Bro.	10	12	9	4	NT	NT	0
20. Van.	4	10	11	4	NT	NT	NT
21. Hes.	17	13	8	4	NT	NT	NT
22. Bla.	11	7	5	2	NT	NT	NT
23. Thom.	11	8	2	0	NT	NT	NT
14 other SLE	0-2	0-6	0-7	0-8	0-5	0	0
Two lupoid hepatitis	2-3	6-8	11-13	16-21	1-8	0-2	NT
10 drug-SLE¶	0-2	0-2	0-2	0-1	NT	NT	NT
15 RA	0	0	0	0	NT	NT	NT
Four polymyositis	0	0	0	0	NT	NT	NT
26 AHA¶	0	0	0	0	0	0	NT
Seven miscellaneous diseases**	0-6	0-1	0	0	NT	NT	NT
14 normals	0	0	0	0	0	0	0

\* Lysosomes were tested at a protein concentration of 25 µg/ml.

‡ All values represent percent CF after correction for anticomplementary effects of lysosomes alone and of serum alone.

§ NT, not tested.

|| Seven of the 14 patients were tested.

¶ Drug-SLE, drug-induced "lupus" syndrome; AHA, autoimmune hemolytic anemia.

\*\* Miscellaneous rheumatic disorders including Wegener's granulomatosis (two cases), polymyalgia rheumatica (two), Henoch-Schönlein purpura (one), chronic glomerulonephritis (one), and viral hepatitis with polyarthritis (one).

were used as antigen, the well was completely filled at least 6 h ahead of the serum wells and then refilled at the time of addition of serum. Lysosomes were used at a concentration of 1,000-1,500 µg/ml. Diffusion occurred during 24-48 h at room temperature.

### Patients

Sera from patients had been stored at -20°C before study. Pleural and joint fluids were examined, however, as fresh specimens. SLE cases were selected only on the basis that clinical and laboratory evidence clearly supported the diagnosis and that sufficient serum was available

for study. Some of these patients had more limited forms of SLE such as thrombocytopenic purpura with positive LE cell test and antinuclear antibody (ANA), or Coombs-positive hemolytic anemia associated with strongly positive ANA, joint symptoms, and Raynaud's phenomenon. The rheumatoid arthritis (RA) patients included had definite or classical RA. The procaine amide-induced lupus patients exhibited diagnostic features previously described (36). Cases listed as autoimmune hemolytic anemia did not have clinical or laboratory features of SLE; most were idiopathic and some were associated with lymphoproliferative disease. All of these patients were from the University of

## Miscellaneous

Rabbit antiserum to human lysosomes was prepared by two immunizations at 3-wk intervals with a total of 500  $\mu$ g of lysosomal protein in complete Freund's adjuvant in footpads and intramuscular sites. The antiserum used in these studies was obtained 10 days after the last injection.

Purified rheumatoid factor (RF), generously provided by Dr. George Abraham, had been isolated from a patient with Felty's syndrome with an exceptionally high latex titer (1/100,000). After fractionation of the serum on Sephadex G-200, proteins eluting in the first peak were concentrated and passed through another Sephadex G-200 column under acid-dissociating conditions. The first peak contained potent IgMK RF exhibiting monoclonal properties in polyacrylamide gel electrophoresis and isoelectric focusing.

In SLE patients antibodies to native or single-strand DNA were measured by a Farr-type radioimmunoassay as described (37) but with native or heat-denatured *E. coli* [ $^3$ H]DNA as antigens.

## RESULTS

### Immunologic reactions of SLE sera with lysosomes

**CF tests.** Lysosomes were tested at 1, 5, 25, and 125  $\mu$ g protein/ml.<sup>4</sup> CF proved to be most efficient at 5  $\mu$ g/ml and particularly at 25  $\mu$ g/ml (Fig. 2), and with SLE serum dilutions in the 1:40 to 1:2,560 range (Table I). Among the 37 SLE patients tested, serum from at least one date of bleeding in 23 patients (62%) gave positive CF reactions (Table I). CF reactions were negative at all dilutions tested with sera from 14 normal donors, 10 patients with drug-induced lupus syndrome (due to procaine amide in nine, hydralazine in one), 15 patients with RA (12 seropositive, three seronegative), four patients with polymyositis, 26 patients with autoimmune hemolytic anemia (not associated with SLE), and a small group of miscellaneous rheumatic disorders (Table I, Fig. 2). Two cases of chronic active ("lupoid") hepatitis with positive ANA gave moderately positive CF reactions with lysosomes (Table I).

Rabbit antilyosome serum produced CF at comparable lysosome and serum concentrations. Lysosomes from six different group O donors gave positive CF reactions of generally similar strength with either rabbit or SLE sera. Entirely comparable CF reactions were obtained with lysosomes prepared by the alternate procedure (see Methods). By gel filtration on Sephadex G-200 the antilyosomal CF activity of two SLE sera eluted with IgG globulins (Fig. 3).

The theoretical possibility arose that the presence of RF in certain SLE or RA sera might interfere with

<sup>4</sup>Lysosome concentrations indicated here and in the figures and tables are initial concentrations. Final concentrations in the CF reaction mixtures are 1/3 the values listed (see Methods).

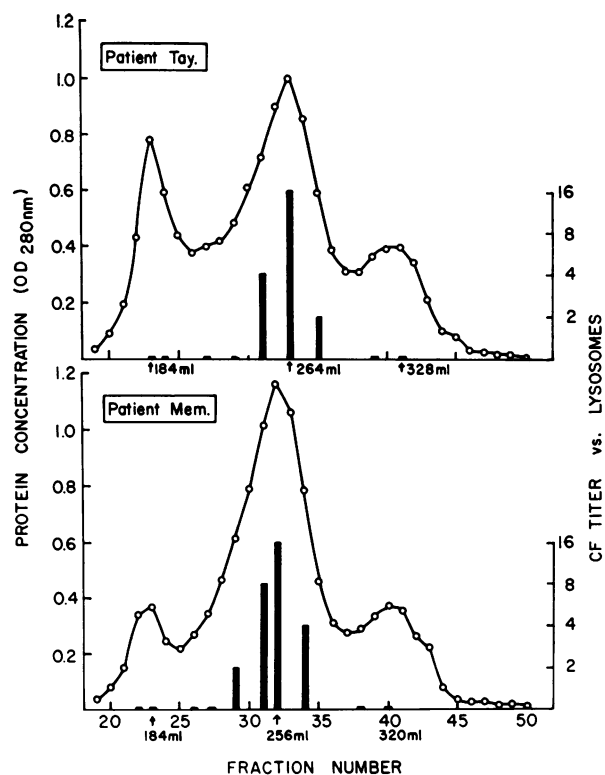


FIGURE 3 Fractionation of antilyosome CF activity in two SLE sera by gel filtration on Sephadex G-200 column. Serial dilutions (1/2-1/16) of fractions designated by black bars were tested against lysosomes at a concentration of 25  $\mu$ g protein/ml.

CF reactions by IgG antilyosome antibodies and thus mask or reduce positive titers. When latex titers for RF were measured in all of the SLE sera listed in Table I, no correlation was apparent between elevated latex titers and weak or absent antilyosome CF reactions. As a more direct test of this issue, purified IgM RF (see Methods) was added to two latex-negative SLE sera: Tay,<sup>5</sup> possessing strong antilyosome reactivity, and Aik., exhibiting only moderate reactivity (see Table I). Control aliquots received buffer alone. Latex titers were raised to 1/5,120 in both RF-treated sera but there was no change in CF reactions at any serum dilution, compared to the controls.

**Gel precipitin reactions.** Seven of 21 SLE sera examined by immunodiffusion against lysosome preparations demonstrated a single precipitin arc close to the antigen well (Table I). This reaction is illustrated in Fig. 4 by serum Kli. A precipitin line against lysosome granules is clearly visible with undiluted serum and faintly with 1:4 serum. The precipitin line between lysosomes and SLE serum Kli. was closer to the antigen

<sup>5</sup>Sera are identified by abbreviation of patient's name.



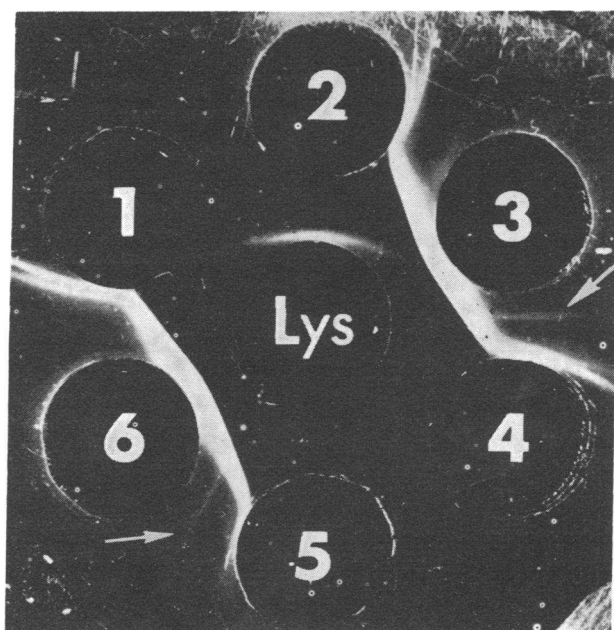


FIGURE 4 Immunodiffusion analysis (0.4% agarose) of SLE sera and rabbit antilyosome serum against human leukocyte lysosomes, 1.5 mg protein/ml (center well). Wells 1 and 5: SLE serum Tay., undiluted and 1:4, respectively. Wells 2 and 4: SLE serum Kli., undil. and 1:4, respectively. Wells 3 and 6: rabbit anti-lysosome serum, 1:4.

(lysosome) well than the much stronger precipitin line(s) obtained with rabbit antilyosome serum (Fig. 4), suggesting that different antigenic components are detected by the SLE and rabbit immune sera by this method. SLE serum Tay. (9/68 bleeding) shows no reaction (Fig. 4), but an earlier pretreatment serum was clearly positive (see Table IV, below). Only those SLE sera exhibiting the strongest CF reactions also showed gel precipitin reactions with lysosomes (Table

I). The faint precipitin lines between the rabbit anti-serum and the SLE sera (arrows, Fig. 4) are discussed below.

**Characterization of reactive lysosomal components.** Multiple aliquots of lysosomes (25  $\mu$ g/ml) were centrifuged at 12,330–40,000  $g$  for variable periods of time. Supernates and uncentrifuged lysosomes were compared in CF reactions with four SLE sera representing strong, moderate, and weak CF reactivity. As shown in Table II, a major loss of the original CF antigenic activity of the lysosomes with SLE sera was observed after centrifugation at 40,000  $g$  for 3 h. Smaller losses of antigenic activity were observed with shorter or slower centrifugations (serums Tay. and Kli.).

Lysosomes at concentrations of 850  $\mu$ g/ml or more ( $>150$  times the concentration capable of giving CF reactions with SLE serum) gave no reaction in the diphenylamine assay for DNA. This indicates that  $<5$   $\mu$ g/ml of DNA is present in a typical undiluted lysosome preparation and  $<0.15$   $\mu$ g/ml in the dilute lysosome suspensions (25  $\mu$ g/ml) which gave optimal CF reactions. Similarly, in two lysosome preparations containing 1,170 and 550  $\mu$ g protein/ml, respectively, the measured (29) RNA content was 20  $\mu$ g/ml. This implies an RNA content of 0.017–0.036  $\mu$ g/ml when such lysosome preparations are diluted (25  $\mu$ g/ml) for CF assays.

Furthermore, as shown in Table III, the capacity to react in CF assays with SLE sera was not affected by prior treatment of the lysosomes with high concentrations of DNase or RNase sufficient to cleave nearly all of the added radioactive DNA or RNA serving as monitors of nuclease activity.

Fig. 5 shows the results of gel precipitin reactions of lysosomes and DNA with two different SLE sera, one of which (Tay., well 4) reacts with both DNA (well 3) and lysosomes (well 1). After extensive absorption with DNA, serum Tay. (well 5) no longer reacts with DNA

TABLE II  
Sedimentation of Lysosomal Antigens Reactive in CF with SLE Sera

Test antigen	Percent CF with SLE serum:								
	Tay.			Kli.		Aik.		Cer.	
	1/640	1/1,280	1/2,560	1/640	1/2,560	1/640	1/2,560	1/640	1/2,560
		%		%		%		%	
Whole lysosomes*	35	38	45	25	31	20	25	11	16
SN‡ (12,330 g, 60 min)	—§	26	17	—	—	—	—	—	—
SN (40,000 g, 20 min)	—	17	13	—	—	—	—	—	—
SN (40,000 g, 60 min)	10	11	7	10	10	—	—	—	—
SN (40,000 g, 180 min)	6	6	3	4	2	5	5	4	3

\* Uncentrifuged lysosome preparation.

† SN, supernate after centrifugation at  $g$  force indicated.

§ Not tested.



TABLE III  
Effect of DNase and RNase Treatment on Reactivity of Lysosomes with SLE Sera

Test material*	Prior treatment	cpm remain- ing (acid ppt)†	Percent CF with:‡				
			SLE serum Tay.		SLE serum Mem.		Rabbit antibody
			1/320	1/1,280	1/320	1/1,280	
			%		%		%
Lysosomes + [ <sup>3</sup> H]DNA	Diluent	8,659	51	50	9	18	48
Lysosomes + [ <sup>3</sup> H]DNA	DNase	126	49	48	10	17	47
Sucrose + [ <sup>3</sup> H]DNA	Diluent	10,799	—	—	—	—	—
Lysosomes + [ <sup>14</sup> C]RNA	Diluent¶¶	438	24	48	28	50	49
Lysosomes + [ <sup>14</sup> C]RNA	RNase¶¶	19	28	47	29	49	48
Sucrose + [ <sup>14</sup> C]RNA	Diluent¶¶	454	—	—	—	—	—

\* Lysosomes, 125 µg + 5 µg [<sup>3</sup>H]DNA or 6.4 µg [<sup>14</sup>C]RNA.

† Radioactivity precipitable by 10% TCA after incubation (see Methods).

‡ Lysosomes from prior step diluted to 25 µg/ml for CF tests.

|| Incubation at 37°C, 60 min, in presence of 0.01 M Mg<sup>++</sup> with or without 20 µg DNase (2,460 U/mg).

¶¶ Incubation at 5°C, 60 min, with or without 25 µg RNase (3,095 U/mg).

(well 6) but reacts just as strongly with lysosomes. The second patient's serum (Mend., well 2) gives no visible reaction with DNA (well 3) but forms a precipitin arc with lysosomes which fuses with that given by serum Tay. The precipitin line between wells 4 and 5 is attributable to excess DNA in the absorbed Tay. serum (well 5).

In other studies (not shown) lysosomes and DNA, which had been incubated in parallel with DNase, were tested by immunodiffusion against SLE sera known to give precipitin reactions with both DNA and lysosomes. This treatment abolished the reactions of DNA with these SLE sera but did not affect the reactivity of lysosomes. Similarly, treatment of lysosomes with RNase had no effect on their precipitin reactions with SLE sera.

SLE serum Mor. gave gel precipitin lines against both lysosomes and a partially purified preparation of the nuclear antigen, Sm (30). These appeared to be reactions of nonidentity but this was not conclusive because of marked differences in the diffusion rates of these two antigens. Exhaustive absorption of Mor. serum with the Sm antigen, however, abolished precipitin reactivity with Sm but not with lysosomes, indicating that the precipitin reaction with lysosomes is not attributable to the Sm: anti-Sm system.

**Clinical correlations.** Table IV summarizes retrospective clinical data on a small group of SLE patients on whom the availability of multiple serum samples permitted serial measurements of antilysozyme antibody. In these cases potent antibody, as defined by CF titers greater than 2,560 and positive precipitin reactions, was found during periods of active disease prior to corti-

costeroid therapy or in association with a clinical relapse after steroids had been discontinued (patient Mem.). Among the other SLE patients, tested at a single point in time, many with negative or very low

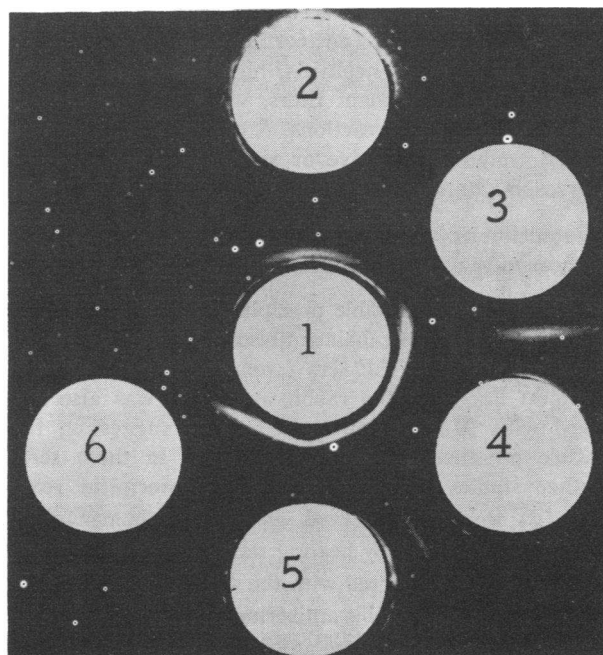


FIGURE 5 Immunodiffusion reactions in 0.4% agarose with the following reactants: Well 1: human leukocyte lysosomes, 1.5 mg protein/ml. Well 2: undiluted SLE serum Mend. Wells 3 and 6: native calf thymus DNA, 1 mg/ml. Wells 4 and 5: undiluted SLE serum Tay., unabsorbed (well 4) and absorbed with native DNA (well 5).

TABLE IV  
*Serial Studies of Antilyosome Antibodies in Selected SLE Patients*

Antilyosome antibody								
		CF reactions*		Precipitin reaction	Active nephritis	Peripheral vasculitis	Arthritis, serositis	Therapy
Patient	Date	Highest percent	Titer					
Tay.	1-9-67	53	10,240	+	++	+	0	None
	9-11-68	11	640	0	0	0	0	Steroids‡
	11-6-68	13	160	0	0	0	0	Steroids‡
Mor.	1-14-69	68	>2,560	+	++	+	NA§	None
	1-20-69	17	2,560	0	++	+	NA	Steroids
	3-28-69	16	2,560	0	±	±	NA	Steroids
Kli.	3-19-68	31	10,240	+	++	++	++	None
	3-25-69	10	640	0	0	0	0	Steroids
Mem.	8-28-68	16	2,560	0	0	0	0	Steroids
	2-28-70	31	10,240	+	+	+	NA	None

\* CF results are listed as highest percent CF at any serum dilution tested and as titer, i.e., highest serum dilution producing at least 10% CF.

† Systemic corticosteroids, usually prednisone.

§ NA, data not available.

titers of antilyosome antibody had been effectively treated with steroids and were clinically inactive at the time of study. On the other hand, there were several patients in the series (on corticosteroids) who still had active SLE with nephritis, high anti-DNA levels, and depressed complement titers, with only modest or negative antilyosome reactions. A consistent correlation between antibody to native or single-strand DNA and antilyosome antibodies was not found.

#### Detection of lysosomal antigen in serum and other body fluids

A faint but reproducible precipitin line (arrows, Fig. 4) is visible between rabbit antilyosome serum (wells 3 and 6) and the two SLE sera (wells 4 and 5). A similar precipitin line with the rabbit antiserum was also observed with 10 of 17 other SLE sera, suggesting the possible presence of lysosomal antigen in these sera. Further studies established that such precipitin reactions (a) were not observed with normal human sera; (b) were not altered by heating the SLE serum to 56°C for 30 min; (c) occurred with the 40 % ammonium sulfate fraction of the rabbit antiserum, but not with preimmune serum from the same animal nor with rabbit antisera to human gamma globulin or to denatured DNA; (d) were not affected by absorption of the rabbit antiserum with pooled normal human serum; and (e) were not observed when rabbit antilyosome serum was diffused against DNA (native and denatured), soluble nu-

cleoprotein, Sm antigen, or human immunoglobulins, all tested at concentrations similar to that of the lysosomes.

Studies were initiated to clarify the relationship of this antigenic component in SLE sera with lysosomes isolated from normal donors. Lysosomes (1.5 mg/ml) prepared by the standard procedure were divided into two equal aliquots. One was subjected to 10 freeze-thaw cycles; the other (control) aliquot was maintained at 37°C. Both preparations were centrifuged at 31,000 *g* (30 min) and the upper 2/3 supernate (SN) was carefully

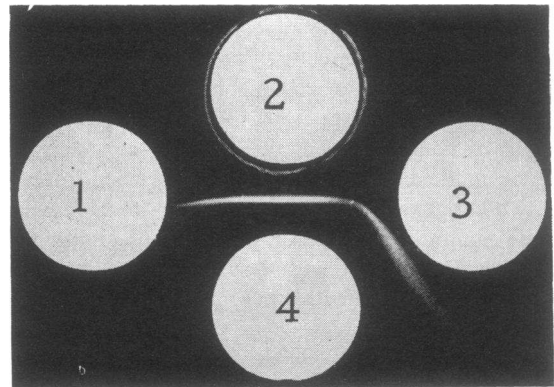


FIGURE 6 Immunodiffusion reactions in 0.4% agarose with the following reactants: Well 1: SN after sedimentation (31,000 *g*, 30 min) of intact granules. Well 2: intact, un-sedimented lysosome granules, 800  $\mu$ g/ml. Well 3: freeze-thaw granule SN after sedimentation (31,000 *g*, 30 min), 475  $\mu$ g/ml. Well 4: rabbit antilyosome serum, 1:4.

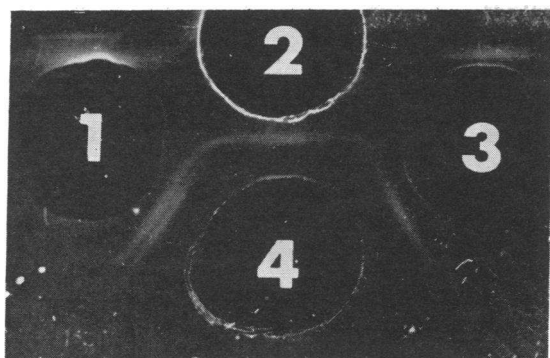


FIGURE 7 Immunodiffusion reactions in 0.4% agarose with the following reactants: Wells 1 and 3: granule supernates from two donors after freeze-thaw disruption and sedimentation at 31,000 *g* for 30 min. Well 2: SLE serum (Wei.), undiluted. Well 4: rabbit antilyosome serum, 1:8.

aspirated. Immunodiffusion analysis (Fig. 6) revealed that a component antigenically identical to a component in the whole ("intact") lysosomes\* was released into the granule SN by freezing and thawing. This was not detectable in the control SN. In Fig. 7 the fusion of the precipitin lines given by the freeze-thaw granule SN and by the lysosomal antigen in the SLE serum indicates their close antigenic relationship. After chromatography on Sephadex G-200 this antigenic activity of the granule SN was identified in the included volume only and was not detectably depleted after centrifugation at 130,000 *g* for 4 h, suggesting that a soluble antigen was involved. The same freeze-thaw granule SN, however, showed no precipitin reactivity with three SLE sera which had given precipitin reactions with intact lysosomes. As shown in Table V, even with the sensitive CF assay there was no reaction between this freeze-thaw SN and a known CF-positive SLE serum; the rabbit antiserum, however, produced a clearly positive reaction, somewhat weaker than with whole lysosomes. These data provide further evidence that the rabbit antiserum and SLE sera react with different lysosomal constituents.

**Lysosomal antigen in various clinical disorders.** The rabbit antilyosome serum was tested (in 0.4% agarose) against sera and other extracellular fluids from patients with various clinical disorders and the results are summarized in Table VI. When present, these precipitin lines always gave reactions of identity with each other (i.e., from one fluid to another or from case to case) and with whole lysosome preparations.

\* "Whole" or "intact" is used to denote lysosome preparations which have not been deliberately disrupted by freeze-thaw treatment or by detergents. It is recognized, however, that a portion of the total lysosomal enzyme content is detectable in fresh lysosome preparations, implying some rupture of granules during isolation.

TABLE V  
CF Reactions Comparing Whole Lysosomes and a Soluble Fraction Obtained by Granule Disruption

Antibody source	Percent CF with:			
	Whole lysosomes (25 $\mu$ g/ml)	Soluble granule supernate*		
		5 $\mu$ g/ml	25 $\mu$ g/ml	100 $\mu$ g/ml
	%	%		
SLE serum Tay. (1/640)	42	1	0	A-C†
Rabbit antilyosome serum (1/1,280)	47	16	22	A-C

\* Obtained by centrifugation at 31,000 *g* for 30 min after 10-cycle freeze-thaw treatment of freshly prepared whole lysosomes (see text).

† The soluble fraction was excessively anticomplementary at this higher concentration.

Another patient not included in Table VI had a transient leukemoid reaction and myalgic syndrome of undetermined etiology. When sera were studied serially, lysosomal antigen could be detected only while there was a significant granulocytosis. In another patient, with SLE, lysosomal antigen appeared during some periods of active disease but could not be correlated with the level of granulocytosis.

## DISCUSSION

This study demonstrates that a significant number of patients with active SLE display CF and gel precipitin reactions with a sedimentable component of lysosome preparations derived from normal human leukocytes. In view of the wide array of autoantibodies characteristic of SLE, considerable effort was made to evaluate the possibility that these reactions were actually due to

TABLE VI  
Immunodiffusion Reactions of Rabbit Antilyosome Serum with Extracellular Fluids of Selected Patients

Clinical disorder	Fluid tested	No. positive
		No. tested
SLE	serum	10/17
	pleural	0/1*
RA	serum	7/10
	synovial	6/6‡
	pleural	1/1‡
Ankylosing spondylitis	synovial	1/1§
Normals	serum	0/30

\* Patient's serum was also negative.

‡ Patients' sera were also positive.

§ Serum was not available on this patient.

antibodies other than antilyosome antibodies reacting with one or more contaminating antigens in the lysosome preparations. Such a contaminating antigen would have had to be present regularly and in substantial concentration to account for consistent gel precipitin reactions on the one hand and strong CF reactions with lysosomes at concentrations as low as 5  $\mu\text{g}/\text{ml}$ <sup>4</sup> on the other (Fig. 2).

Several lines of evidence indicated that these immunologic reactions could not be attributed to DNA:anti-DNA or RNA:anti-RNA reactions. These include (a) the very low concentrations of DNA (< 5  $\mu\text{g}/\text{ml}$ ) and RNA ( $\sim 20$   $\mu\text{g}/\text{ml}$ ) in undiluted suspensions of lysosomes containing 550–1,500  $\mu\text{g}$  protein/ml; (b) the failure of high concentrations of demonstrably active DNase or RNase to affect either CF or precipitin reactions of lysosomes with SLE sera; (c) our ability to absorb anti-DNA antibody from an SLE serum without affecting its reactivity with lysosomes; and (d) the lack of correlation of anti-DNA and antilyosome reactions in individual sera.

Autoantibodies to other subcellular cytoplasmic organelles including mitochondria (8–11), microsomes (9, 10), ribosomes (12–14), and soluble cytoplasmic antigens (15) have been identified by others in SLE sera. It appears unlikely that the reactions of our SLE sera with lysosomes are attributable to antimitochondrial antibodies because (a) mitochondrial structures were rare in our lysosome preparations by electron microscopy (Fig. 1), (b) neither succinic dehydrogenase nor reduced NAD dehydrogenase (cytochrome *c* reductase) activities were detected, and (c) the sedimentation characteristics of our lysosomal antigen are not typical of mitochondria (38). Likewise, the RNase resistance and sedimentation properties of our lysosome antigen suggest that ribonucleoprotein, ribosomes, microsomes, or soluble cytoplasmic constituents are not the antigens responsible for the observed reactivity (see 9, 38, 39). Our inability to detect ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase in lysosomal membranes is evidence against major contamination by fragments of the leukocyte plasma membrane (25). Although we cannot exclude the possibility of a nonlysosomal antigen bound to lysosome membranes, it seems reasonable to ascribe the observed CF and precipitin reactions to the presence of antibodies in the SLE sera reactive with membrane-bound antigen of lysosome granules.

This study, utilizing extensively characterized preparations of human lysosomes, confirms and extends the previous reports of Wiedermann and associates on the occurrence of antilyosome antibodies in SLE (10, 16). The studies of these authors differ from ours in several respects. First, their published data are based largely on CF reactions of SLE sera with cytoplasmic fractions from rat and guinea pig liver and kidney. Secondly, CF

activity was demonstrated by these workers using antigens at protein concentrations 12–20 times higher than in our (25  $\mu\text{g}/\text{ml}$ ) preparations. This might reflect the presence of a relatively large proportion of nonlysosome proteins in their admittedly crude (16) fractions, or a relative inefficiency of heterologous antigens in the CF assay.

The actual frequency of antilyosome antibodies in SLE may not be accurately reflected by the data in Table I and Fig. 2. In many of the present cases serum from the most active or pretreatment phase of the illness was not available. The limited data in Table III suggest that corticosteroid therapy may effectively suppress such antibody.

In addition to the observations on antilyosome antibodies, evidence has been presented for the occurrence of lysosomal antigen in serum, synovial fluid, and pleural fluid of patients with SLE or other inflammatory diseases. This lysosomal antigen, which was identified by gel precipitin reactions with rabbit antiserum to human leukocyte lysosomes, appeared to be antigenically identical to a soluble component which can be released in vitro from intact lysosomes of normal donors. Since this component does not detectably react with SLE sera known to give precipitin or CF reactions with intact lysosomes, the naturally occurring lysosomal antigen appears to be either a distinct substance (e.g., a lysosomal enzyme rather than membrane antigen) or a membrane antigen in a degraded form.

The appearance of this lysosomal antigen in a variety of inflammatory fluids and in the serum of a non-SLE patient during a transient neutrophilic leukemoid reaction is consistent with its origin from granulocytes in vivo. Artifacts release of lysosomal antigen as a result of cell damage during collection of blood or fluids with high granulocyte counts was also considered. While this possibility cannot be dismissed, it seems unlikely in view of the uniform absence of the antigen in normals and its occurrence in SLE and RA patients with normal or reduced peripheral blood granulocytes. It is probable that a variety of subcellular antigens may be released at sites of tissue injury of different etiologies (40). The presence of DNA (41, 42) and of lysosomal enzyme activities (43, 44) in pathologic joint fluids and of DNA in the serum of patients after corticosteroid therapy or open heart surgery (42) are thought to be further examples of this process. Thus, the occurrence of soluble lysosomal antigen in SLE serum (or other body fluids) may well prove to be a normal aspect of inflammation.

The present investigations indicate, however, that what is abnormal in SLE is the formation of antilyosome antibody. This, in turn, raises the question of the possible significance of such antibody. Earlier studies provided in vitro evidence for a protective effect of

antilyosome antibodies, i.e., lysosome stabilization (16, 45). Wiedermann, Hirschhorn, Weissmann, and Miescher (16), moreover, suggested that the autoantibody response to lysosomes may be a physiologic reaction to tissue injury. Yet, in the present studies, antibody to human lysosomes was not detected in sera of selected groups of patients with a variety of other rheumatic or autoimmune disorders except lupoid hepatitis (Table I).

To date, we have not demonstrated in SLE sera the presence of the membrane-associated (sedimentable) lysosomal antigen with which the human antilyosome antibodies react. Such material may remain in inflamed tissues or be rapidly cleared from body fluids by phagocytes. It is conceivable that in SLE patients possessing circulating antibody to lysosomes, leukocyte breakdown at sites of inflammation could result in *local* formation and deposition of lysosome: antilyosome complexes with potential for perpetuation of tissue injury. The relationship, if any, of such antibodies in SLE patients to the other, soluble lysosomal antigen (i.e., that which is reactive with rabbit antiserum and antigenically similar to a nonsedimentable component released from intact granules) is not apparent at this time.

A final point of interest was the possible association of antilyosome antibody with leukopenia, thrombocytopenia, or Coombs-positive hemolytic anemia in SLE patients. In vitro studies had suggested that the membranes of lysosomes, leukocytes, and erythrocytes share certain antigenic determinants (45-48) and that heterologous antilyosome antibody may injure intact granulocytes under experimental conditions (46). Retrospective analysis of our patients' records did not demonstrate any of these associations, in agreement with the conclusion of Wiedermann and Miescher (10).

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