Journal of Clinical Investigation
Vol. 42, No. 6, 1963

SYSTEMIC LUPUS ERYTHEMATOSUS. CRYOPRECIPITATION OF SERA

By CHARLES L. CHRISTIAN,* WENDELL B. HATFIELD,† AND P. HARVEY CHASE‡

(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York, N. Y.)

(Submitted for publication December 28, 1962; accepted February 13, 1963)

Sera of normal human subjects, when refrigerated (0 to 4° C), form negligible quantities of precipitate. A variety of pathologic states have been associated with serum cryoprecipitation: myelomatosis, Waldenström's macroglobulinemia, lymphosarcoma, kala azar, bacterial endocarditis, rheumatoid arthritis, periarteritis, and systemic lupus erythematosus (SLE) (1). Lerner, Watson, and Barnum suggested the term "cryoglobulins" for cold-precipitable elements of sera and established quantitative criteria for their studies (*2*, 3). When serum cryoproteins have been characterized, the majority have been homogeneous in electrophoretic and sedimentation studies. Cryoproteins of myeloma sera usually consist of the respective paraproteins. The cryoproteins separating from sera with "rheumatoid factor" (RF) properties have been inhomogeneous, consisting in part of the RF (4–6).

This report concerns studies of SLE sera that precipitated at 0 to 4° C. In 12 SLE sera, the following were observed: *1*) the association of cryoprecipitation with active disease manifestations, *2*) inhomogeneity of cryoprecipitates consisting in part of *7* and 19 S γ-globulin, and *3*) the requirement for cryoprecipitation of a heat-labile (30 minutes at 56° C) component that could be supplied by normal human sera and the identification of the heat-labile substance as a recently characterized component of serum complement.

MATERIALS AND METHODS

1. Blood samples obtained from patients in the Edward Daniels Faulkner Arthritis Clinic and the Presbyterian Hospital were allowed to clot in a 37° C water bath. Sera were separated from clotted blood by brief centrifugation at room temperature. The studies to be described were performed on fresh, unfrozen sera. See Table I for a summary of clinical and laboratory features of the 12 patients included in the present study.

2. Serum reagents for titration of complement components were prepared by previously reported methods

* Investigator of the Health Research Council of the City of New York under contract I-178. John and Mary R. Markle Foundation Scholar in the Medical Sciences.

† Trainee under U. S. Public Health Service grant 2A-5040 from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

‡ Fellow, The National Foundation, New York, N. Y.

TABLE I

*Summary of clinical and laboratory features of patients in the present study**

Patient	Systemic manifestations					Anemia, <10 g hemoglobin	Leukopenia, <5,000 leukocytes	LE prep. positive	Rheumatoid factor present	Status in December, 1962		
	Arthritis	Serositis	Skin lesions	Nephritis	Raynaud's					Moderate remission	Active SLE	Dead
E.T.	x	x	x	x	x	x	x	x				x
M.H.	x	x	x	x	x		x	x	x			x
E.S.	x		x					x		x		
H.M.	x		x	x		x		x		?	?	?
R.J.	x	x		x		x	x	x		?	?	?
J.C.	x			x			x					x
L.C.	x	x	x	x		x	x	x		x		
R.W.	x	x		x		x	x	x		x		
H.H.	x	x				x		x		x		
J.R.	x		x	x		x	x	x				x
J.H.	x				x		x		x	?	?	?
C.M.	x	x	x	x		x	x	x				x

* LE = lupus erythematosus; SLE = systemic lupus erythematosus.

TABLE II

Cryoprecipitation of SLE sera. The effect of heating (30 minutes at 56° C) and reconstitution of precipitability by normal human serum reagents

Tube number: Reagents mixed and incubated*	1	2	3	4	5	6	7	8	9	10	11
Normal serum, 1 ml				Untreated	Heated	Zymosan-treated	Ammonia-treated	Immune ppt. absorbed	Euglobulin (dialysis)	Supernatant fluid (dialysis)	Purified C'_1
SLE serum, 1 ml	Untreated	EDTA 0.01 M	Heated	Heated	Heated	Heated	Heated	Heated	Heated	Heated	Heated
				precipitate nitrogen, μg/ml serum							
SLE sera											
E.T.	35		12	37	10	35	40				
C.H.	12		2	18	2	15	13	2	12	2	
E.S.	24		2	33	2	35	37	2	32	0	
H.M.	30		18	33	17	25	23	18	37	13	
R.J.	63		8	65	7	62	55	8	63	5	
J.C.	27		5	30	7	32	32	5	22	3	45
L.C.	24	15	5	12	5	15	10	5	25	5	28
R.W.	24	28	6	22	7	22	17	5	22	5	24
H.H.	15	22	2	8	2			2	10	2	13
J.R.	20	28	2	15	2			3	15	3	18
Mean ppt. yield, %	100	112	23	100	23	101	95	21	100	16	116

* For 48 hours at 0 to 4° C.
† Relative to precipitate yield from untreated SLE sera.

(7) and quantitated as 50% hemolytic units. The following reagents were used: serum heated at 56° C for 30 minutes, serum absorbed with Zymosan (1.35 mg per ml of serum), ammonia-treated serum, euglobulin precipitate, and supernatant fluid (dialysis method). Partially purified C'_1 was prepared by the method of Lepow, Ratnoff, Rosen, and Pillemer (8). 11 S material was prepared from normal human sera as described by Müller-Eberhard and Kunkel (9).

3. Cryoprecipitation was quantitated by placing measured samples of test sera into calibrated, 8-ml, conical centrifuge tubes. After refrigeration at 0 to 4° C for 48 hours, the tubes were centrifuged at 4° C and washed three times with cold isotonic saline. The washed precipitates were dissolved in 2 drops of 1 N NaOH and diluted up to 3 ml with water. Nitrogen contents of samples were estimated by the Folin-Ciocalteu reaction (10). The colorimetric method was standardized with a preparation of human γ-globulin whose nitrogen content was determined by Kjeldahl analysis.

4. The characterization of a heat-labile component required for cryoprecipitation was obtained by adding normal serum reagents to SLE sera heated at 56° C for 30 minutes. With all reagents other than the 11 S material and purified C'_1, the equivalent of 1 ml of treated normal serum was added to 1 ml of heated SLE sera, and the incubation and quantitation were carried out as described above.

5. Sedimentation studies of dissolved cryoprecipitates (0.15 M NaCl, 0.01 M phosphate buffer at pH 7.4) were performed in a model E Spinco analytical ultracentrifuge.

6. The technique of Grabar and Williams was utilized in immunoelectrophoretic studies (11).

RESULTS

Table II presents data obtained from a study of 10 SLE sera demonstrating significant cryoprecipitation. The values for precipitate nitrogen ranged between 12 and 63 μg nitrogen per ml,

TABLE III

Effect of purified C'_1 in reconstituting cryoprecipitability of two heated SLE sera*

SLE sera (1-ml samples)	Normal serum reagents added		Precipitate nitrogen
		ml	*μg/ml SLE sera*
J. C. Untreated			27
Heated*			5
	Untreated serum:	1	20
	Heated* serum:	1	4
	Purified C'_1†:	0.1	4
		0.25	22
		0.5	41
		0.75	48
		1.0	48
R. J. Untreated			38
Heated*			5
	Untreated serum:	1	39
	Heated* serum:	1	4
	Purified C'_1†:	0.25	34
		0.75	32
		1.0	32
		0.25‡	6

* 30 minutes at 56° C.
† 500 U C'_1 per ml.
‡ Heated* after addition of purified C'_1.

TABLE IV

Effect of 11 S material in reconstitution of cryoprecipitation of SLE sera

Tube number:	1	2	3	4	5	6	7	8	9	10	11
Reagents mixed and incubated*											
SLE serum, 1 ml	Untreated	†	†	†	†	†	†	†	†	†	†
11 S material, μg nitrogen added			47	14	14‡	20	20‡	50	50‡	100	100‡
					precipitate nitrogen, μg/ml serum						
SLE sera											
C.M.	75	8				51	16	74	11	83	10
E.T.	44	4	89	41	8						
J.H.	110	9	88								
J.R.	20	5	38								

* For 48 hours at 0 to 4° C.
† Heated 30 minutes at 56° C.
‡ Heated* after addition of 11 S material to serum.

equivalent to 7 to 38 mg per 100 ml. In all sera studied, heating at 56° C for 30 minutes significantly reduced cryoprecipitation, and the addition of untreated normal human serum to heat-inactivated SLE sera partially or completely reconstituted cryoprecipitation. Zymosan-absorbed normal human serum (relatively deficient in C'_3), ammonia-treated serum (relatively deficient in C'_4), and the euglobulin fraction (relatively deficient in C'_2, C'_3, and C'_4) all reconstituted cryoprecipitation. Reagents lacking C'_1 (heated normal human serum, immune precipitate-absorbed serum, and the supernatant fluid after removal of the euglobulin fraction) did not result in significant precipitation when added to heated SLE sera. Further evidence that C'_1 might be the heat-labile factor required for cryoprecipitation derived from the effect of purified C'_1 in reconstituting precipitation of heated SLE sera. More detailed studies of purified C'_1 in which graded amounts of this reagent were added to two heated SLE sera are summarized in Table III. Although purified C'_1 induced more precipitate than was obtained from untreated SLE serum in one subject (J.C.), a plateau effect was reached for both sera after addition of 0.25 to 0.5 ml purified C'_1; larger amounts did not result in significant increases in precipitate formation. When heated serum of Subject R.J. was reheated after addition of purified C'_1, the reconstituting property of the latter was lost.

Although the observations so far presented are compatible with the identification of the heat-labile component as C'_1, the finding that disodium ethyl-

TABLE V

Reconstitution of cryoprecipitation of a heated SLE serum by the addition of varying amounts of 11 S material

Tube number:	1	2	3	4	5	6	7	8	9	10	11	12	13
Normal serum (M.F.), ml													
Untreated	1												
Heated*		1	1										
SLE serum (E.T.), ml													
Untreated				1									
Heated*					1	1	1	1	1	1	1		
Supernatant fluid†												1	1
11 S material													
Nitrogen added, μg			45			9	18	45	90	180			45
Nitrogen added,‡ μg											45		
Precipitate nitrogen, μg per ml	3	3	4	44	9	8	67	125	126	116	12	4	52

* Thirty minutes at 56° C.
† After removal of cryoprecipitate from untreated serum.
‡ Heated* after addition of 11 S material to serum.

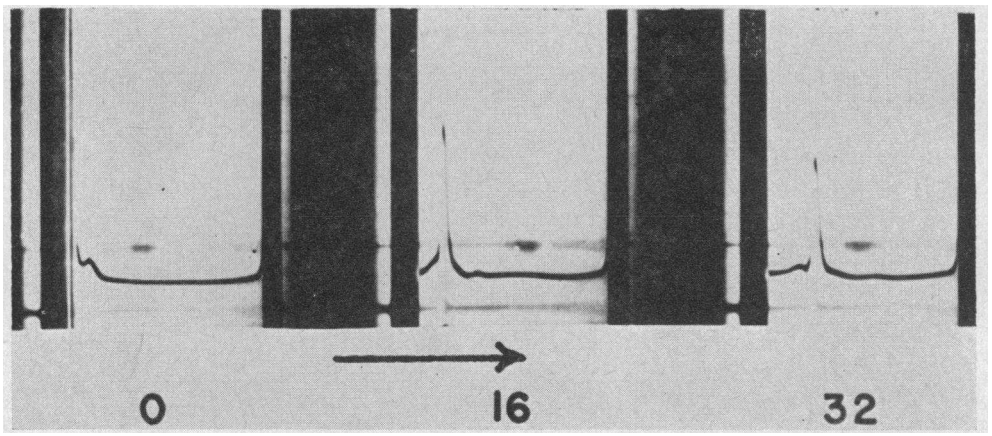

FIG. 1. ANALYTICAL ULTRACENTRIFUGE PATTERN OF 11 S MATERIAL. Sedimentation was at 47,660 rpm in direction of arrow; exposure intervals, in minutes, are indicated. Small quantities of components with S constants of approximately 19 and 7 are present.

enediaminetetraacetic acid (EDTA) failed to prevent cryoprecipitation is evidence to the contrary. [All evidence indicates that calcium is required for the interaction of C'_1 with immune complexes (12–14).] In 3 of the 4 SLE sera studied with EDTA, the chelating agent resulted in increased cryoprecipitation over that observed with untreated sera. A newly characterized component of hemolytically active complement (11 S component) has properties in common with the

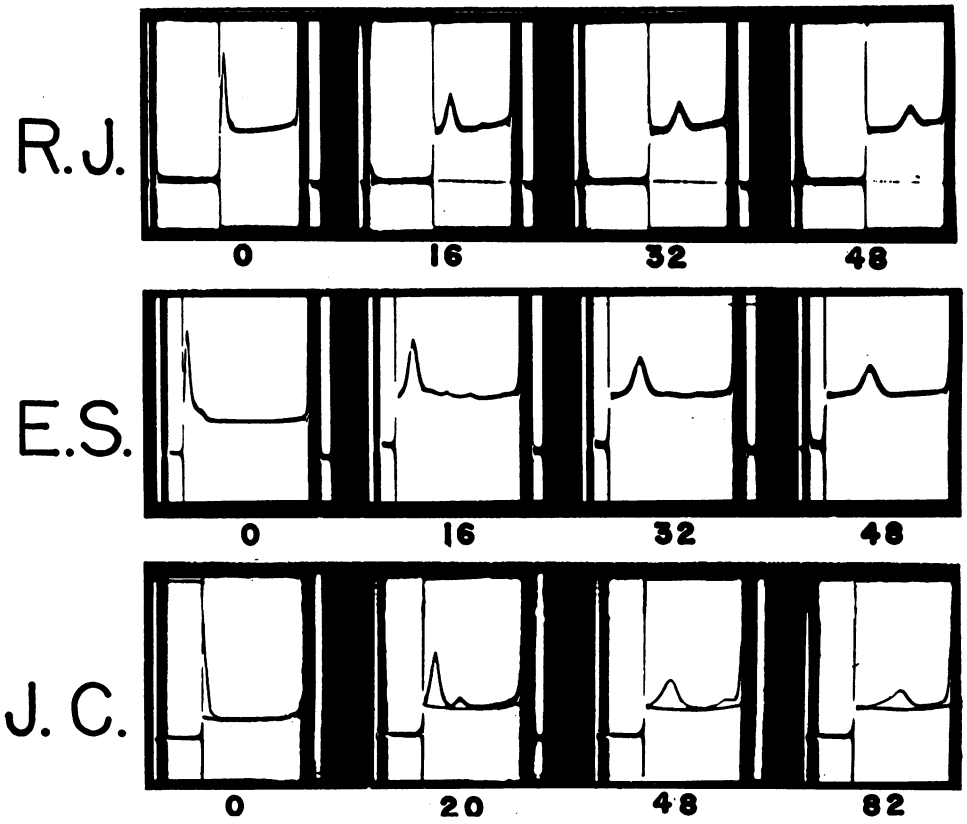

FIG. 2. ANALYTICAL ULTRACENTRIFUGE PATTERNS OF 3 PURIFIED CRYOPROTEINS. Sedimentation was at 46,660 rpm. Exposure intervals, in minutes, are indicated. The major component in each sample has an S-constant of 6 to 7. More rapidly sedimenting components are also seen.

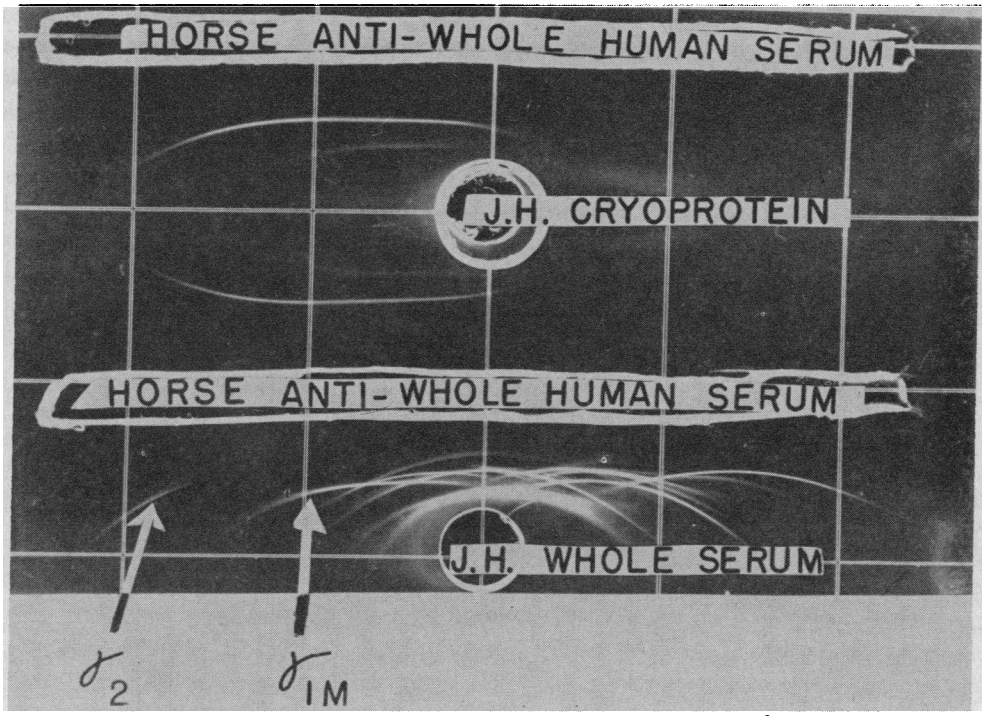

FIG. 3. IMMUNOELECTROPHORETIC EVIDENCE OF THE PRESENCE OF γ_2- AND γ_{1M}-GLOBULINS IN PURIFIED SLE CRYOPROTEIN.

normal serum factor required for reconstitution of cryoprecipitation of heated SLE sera: *1*) heat lability, *2*) coexistence with C'_1 in standard complement reagents, and *3*) normal or increased effect in the presence of EDTA (9, 15).

Table IV summarizes studies in which small amounts of 11 S material reconstituted cryoprecipitation of 4 heated SLE sera (Figure 1 illustrates a sedimentation pattern of the 11 S material used). When 2 sera (C.M. and E.T.) were reheated after addition of the 11 S material, increased precipitation did not occur. Other studies with 11 S material are presented in Table V. Amounts of 11 S material varying between 9 and 180 μg nitrogen were added to samples of a heated SLE serum. A plateau effect was reached with 45 μg nitrogen; a further, fourfold increase in 11 S material did not increase cryoprecipitation. Forty-five μg 11 S material did not induce precipitation when added to normal human serum. When 11 S material was added to the supernatant fluid of untreated SLE serum after removal of cryoprecipitate, further precipitation was induced, indicating that for this serum, the 11 S material was the limiting reactant required for cryoprecipitation.

Analytical ultracentrifuge studies were performed on purified cryoproteins obtained from 8 SLE sera. In most cases, a small residue of precipitate was not soluble on warming to 37° C, so that materials in solution may not have reflected the total composition of cryoproteins. Sedimentation patterns of 3 cryoproteins are illustrated in Figure 2. All of the cryoproteins examined demonstrated predominantly 7 S material with varying quantities of more rapidly sedimenting components.

Purified cryoproteins from 4 SLE sera were studied by immunoelectrophoresis with horse antihuman antiserum. Two antigens, γ_2- and γ_{1M}-globulins, were consistently demonstrable (see Figure 3).

DISCUSSION

Occasional reference has been made to the association of SLE and cryoproteinemia. Coburn and Moore, in an electrophoretic study of certain SLE sera, noted a dense precipitation in the part of the Tiselius tube containing globulin (16). Barr, Reader, and Wheeler found significant cryoproteinemia in 8 of 121 random human sera (17). Three of these 8 were SLE sera (total of 6 SLE

sera included in the 121 studied). Waldenström referred to a single case of SLE that exhibited cryoproteinemia (18).

The magnitude of cryoprecipitation reported here (7 to 38 mg per 100 ml) is equivalent to grade 2 cryoglobulinemia in Lerner's studies. Cryoproteinemia of this degree is rarely encountered. Our group of patients is not a random sampling of SLE patients. Screening tests were applied to virtually all hospitalized patients, although the outpatient population was incompletely surveyed. All patients with cryoproteinemia, however, showed signs of active SLE. During the 3 years of study, 18 of 33 sera examined contained at least 10 μg nitrogen per ml serum. Of these patients, 8 are known to have died; significant renal disease was present in 15; only 5 patients had Raynaud's symptoms.

The requirement of a component of complement (11 S material) for cryoprecipitation may be significantly related to the nature of the reactants participating in precipitate formation and to the pathogenesis of SLE. Heat-labile cryoprecipitation in another report concerned a patient with "essential cryoglobulinemia" (19).

Current knowledge suggests that the 11 S component of complement reacts with immune complexes or with aggregates of γ-globulin. [γ-Globulin aggregates share other properties with immune complexes: reactivity with rheumatoid factor (20, 21) and the capacity to induce cutaneous inflammatory lesions (22, 23).] The precipitation of otherwise soluble components of SLE sera by 11 S material is apparently analogous to the action of complement in rendering soluble immune complexes insoluble (24–26). The presumed aggregates in SLE sera that are reactive with 11 S material might be either the products of nonspecific aggregation of γ-globulin, or, conceivably, immune complexes. The evidence of this report does not clearly support one of the above possibilities to the exclusion of the other. The γ-globulin constituents of dissolved cryoproteins could represent antibodies dissociated from immune complexes, but the antigen of the hypothetical immune complex was not demonstrable. One could postulate that the immune complexes might be anti-γ-globulin antibodies in combination with autologous γ-globulin. Such a basis has been suggested for the intermediate complexes of γ-globulin observed in certain rheumatoid sera (27).

Evidence of *in vivo* fixation of complement in SLE derives from demonstrations of low total serum complement (28–30). A study of components of complement revealed depressions of the 11 S material as well as decreases in other components in SLE sera (31). Immunoelectrophoretic studies of SLE sera demonstrated marked reduction of a globulin, B_{1c}-globulin, subsequently shown to be a component of complement (32, 33). Immunofluorescent studies demonstrated the presence of B_{1c}-globulin in renal lesions of SLE (34). In another immunofluorescent study, complement (defined as human serum components complexing with immune complexes) was demonstrated in renal lesions of SLE (35).

Our observations are compatible with the thesis that either antigen-antibody complexes or nonimmune aggregates of γ-globulin exist in the sera of SLE patients. Tissue injury by such complexes, perhaps with the mediation of serum complement, could account for the varied pathologic features of SLE.

SUMMARY

Selected sera of systemic lupus erythematosus (SLE) demonstrated cryoprecipitation that was dependent on a heat-labile serum factor. The heat-labile component was present in normal human sera and was identified as a component of complement (11 S material). These observations, in addition to previous studies of complement in SLE, are consistent with the thesis that immune complexes exist in SLE sera.

REFERENCES

1. Mackay, I. R., N. Eriksen, A. G. Motulsky, and W. Volwiler. Cryo- and macroglobulinemia. Electrophoretic, ultracentrifugal and clinical studies. Amer. J. Med. 1956, **20**, 564.
2. Lerner, A. B., and C. J. Watson. Studies of cryoglobulins. I. Unusual purpura associated with the presence of a high concentration of cryoglobulin (cold precipitable serum globulin). Amer. J. med. Sci. 1947, **214**, 410.
3. Lerner, A. B., C. P. Barnum, and C. J. Watson. Studies of cryoglobulins. II. The spontaneous precipitation of protein from serum at 5° C in various disease states. Amer. J. med. Sci. 1947, **214**, 416.
4. Christian, C. L. A study of rheumatoid arthritis sera: comparison of spontaneous precipitates and

gamma globulin-induced precipitates. Arthr. and Rheum. 1959, **2**, 289.

5. Epstein, W. V., E. P. Engleman, and M. Ross. Quantitative studies of the precipitation and agglutination reactions between serum of patients with "connective tissue" diseases and a preparation (Cohn fraction II) of human gamma globulin. J. Immunol. 1957, **79**, 441.
6. LoSpalluto, J., B. Dorward, W. Miller, Jr., and M. Ziff. Cryoglobulinemia based on interaction between a gamma macroglobulin and 7S gamma globulin. Amer. J. Med. 1962, **32**, 142.
7. Kabat, E. A., and M. M. Mayer. Experimental Immunochemistry. Springfield, Ill., Charles C Thomas, 1948, p. 117.
8. Lepow, I. H., O. D. Ratnoff, F. S. Rosen, and L. Pillemer. Observations on pro-esterase associated with partially purified first component of human complement (C'_1). Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 32.
9. Müller-Eberhard, H. J., and H. G. Kunkel. Isolation of a thermolabile serum protein which precipitates γ-globulin aggregates and participates in immune hemolysis. Proc. Soc. exp. Biol. (N. Y.) 1961, **106**, 291.
10. Heidelberger, M., and C. F. C. MacPherson. Quantitative micro-estimation of antibodies in sera of man and other animals. Science 1943, **97**, 405 (and **98**, 63).
11. Grabar, P., and C. A. Williams. Méthode permettant l'étude conjugée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines. Application au sérum sanguin. Biochim. biophys. Acta (Amst.) 1953, **10**, 193.
12. Levine, L., K. M. Cowan, A. G. Osler, and M. M. Mayer. Studies on role of Ca^{++} and Mg^{++} in complement fixation and immune hemolysis. J. Immunol. 1953, **71**, 359.
13. Maurer, P. H., and W. Weigle. Persistence of complement in aged sera. J. Immunol. 1953, **71**, 284.
14. Kabat, E. A. Experimental Immunochemistry. Springfield, Ill., Charles C Thomas, 1948.
15. Taranta, A., H. S. Weiss, and E. C. Franklin. Precipitating factor for aggregated γ-globulins in normal human sera. Nature (Lond.) 1961, **189**, 239.
16. Coburn, A. F., and D. H. Moore. The plasma proteins in disseminated lupus erythematosus. Bull. Johns Hopk. Hosp. 1943, **73**, 196.
17. Barr, D. P., G. G. Reader, and C. H. Wheeler. Cryoglobulinemia. I. Report of 2 cases with discussion of clinical manifestations, incidence and significance. Ann. intern. Med. 1950, **32**, 6.
18. Waldenström, J. Pathological globulins and protein synthesis. Exp. Med. Surg. 1954, **12**, 187.
19. Volpé, R., A. Bruce-Robertson, A. A. Fletcher, and W. B. Charles. Essential cryoglobulinaemia. Review of the literature and report of a case treated with ACTH and cortisone. Amer. J. Med. 1956, **20**, 533.
20. Christian, C. L. Studies of aggregated γ-globulin. I. Sedimentation, electrophoretic and anticomplementary properties. J. Immunol. 1960, **84**, 112.
21. Edelman, G. M., H. G. Kunkel, and E. C. Franklin. Interaction of the rheumatoid factor with antigen-antibody complexes and aggregated gamma globulin. J. exp. Med. 1958, **108**, 105.
22. Ishizaka, T., and K. Ishizaka. Biological activities of aggregated gamma globulin. I. Skin reactive and complement-fixing properties of heat denatured gamma globulin. Proc. Soc. exp. Biol. (N. Y.) 1959, **101**, 845.
23. Christian, C. L. Studies of aggregated γ-globulin. II. Effect *in vivo*. J. Immunol. 1960, **84**, 117.
24. Maurer, P. H., and D. W. Talmage. The effect of the presence of complement in rabbit serum on the quantitative precipitin reaction. II. Effect of antigen and antibody precipitation. J. Immunol. 1953, **70**, 435.
25. Weigle, W. O., and P. H. Maurer. The effect of complements on soluble antigen-antibody complexes. J. Immunol. 1957, **79**, 211.
26. Weigle, W. O., and P. H. Maurer. The effect of chemical and physical treatments of sera on the hemolytic activities and fixation of complement nitrogen by antigen-antibody precipitates. J. Immunol. 1957, **79**, 370.
27. Kunkel, H. G., H. J. Müller-Eberhard, H. H. Fudenberg, and T. B. Tomasi. Gamma globulin complexes in rheumatoid arthritis and certain other conditions. J. clin. Invest. 1961, **40**, 117.
28. Vaughan, J. H., T. B. Bayles, and C. B. Favour. Response of serum gamma globulin level and complement titer to adrenocorticotropic hormone (ACTH) therapy in lupus erythematosus disseminatus. J. Lab. clin. Med. 1951, **37**, 698.
29. Elliott, J. A., Jr., and D. R. Mathieson. Complement in disseminated (systemic) lupus erythematosus. Arch. Derm. Syph. (Berl.) 1953, **68**, 119.
30. Williams, R. C., Jr., and D. H. Law IV. Serum complement in connective tissue disorders. J. Lab. clin. Med. 1958, **52**, 273.
31. Morse, J. H., H. J. Müller-Eberhard, and H. G. Kunkel. Antinuclear factors and serum complement in systemic lupus erythematosus. Bull. N. Y. Acad. Med. 1962, **38**, 641.
32. Seligmann, M., and C. Hanau. Étude immuno-électrophorétique du sérum de malades atteints de lupus érythémateux disséminé. Rev. Hémat. 1958, **13**, 239.
33. Müller-Eberhard, H. J., and U. Nilsson. Relation of a β_1-glycoprotein of human serum to the complement system. J. exp. Med. 1960, **111**, 217.
34. Lachmann, P. J., H. J. Müller-Eberhard, H. G. Kunkel, and F. Paronetto. The localization of in vivo bound complement in tissue sections. J. exp. Med. 1962, **115**, 63.
35. Freedman, P., and A. S. Markowitz. Gamma globulin and complement in the diseased kidney. J. clin. Invest. 1962, **41**, 328.