Complement Fixation with Cell Nuclei and DNA in Lupus Erythematosus. (23545)

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Evidence has been presented(1) that the lupus erythematosus (L.E.) serum factor reacted specifically with nuclei and nucleoprotein. It could be readily removed from serum by absorption on nuclei or nucleoprotein and active material could be recovered by elution. Localization on the nuclei was demonstrated by the fluorescent antibody technic as well as by the ability of such nuclei to be phagocytized to form L.E. cells. Similar results were obtained with isolated nucleoprotein. The possibility was raised that the L.E. factor was an antibody; it had the physical properties of a γ-globulin and appeared to react with antiserum to normal γ-globulin. In order to explore this concept further, experiments with complement fixation have been carried out to see if complement is removed in the reaction between the L.E. factor and nuclei. Desoxyribosenucleic acid (DNA) as a major constituent of nuclei also has been studied by this technic. It was felt that a complement fixation reaction specific for this disease or for the L.E. factor might have advantages over the L.E. test clinically as well as furnishing information regarding possible antinuclear antibodies.

Materials and methods. Sera: All sera were stored at 4°C and inactivated in a waterbath at 56°C for 30 minutes prior to use in complement fixation. *Preparation of nuclei*: Calf thymus nuclei were prepared by the method of Mirsky *et al.*(2). The human monocytes were obtained from a patient with acute monocytic leukemia. The cells were suspended in a solution of 0.25 M sucrose and 0.003 M $CaCl_2$, disrupted in a Potter homogenizer, and the nuclei separated by centrifugation. The rabbit leukocyte nuclei were prepared by a similar method from glycogen-induced peritoneal exudates. All nuclei were lyophilized and stored in a desiccator at 4°C. The lyophilized nuclei were suspended in buffer immediately prior to use with gentle mixing. None of the nuclear preparations was anticomplementary in the concentrations used (1 or 2 mg/ml) and suspensions of calf thymocyte nuclei were not anticomplementary even in a concentration of 10 mg/ml. *DNA*: Calf thymus DNA was prepared by the Sevag method and lyophilized.† It was dissolved in a small volume of distilled water with stirring and made to final volume with buffer. Powdered salmon sperm DNA was obtained from the California Foundation for Biochemical Research and was prepared for use similarly. Calf thymus DNA was strongly anti-complementary in concentrations of 500 γ/ml or higher, but was not at 100 γ/ml or lower. It was employed in complement fixation in a concentration of 5 to 100 γ/ml. These concentrations of DNA had no effect on complement titrations. Furthermore, complement fixation was stronger with concentrations of DNA of 5-20 γ/ml than with higher concentrations. *Complement fixation test*: This was performed after the method of Casals and Palacios(3). All dilutions were made in veronal saline buffer containing Ca and Mg ions, pH 7.4. Fresh guinea pig serum stored at –20°C was used as complement. It was titrated prior to use in each test, and was diluted so that 0.5 ml contained 2 units. The test was carried out as follows: 0.25 ml of serum serially diluted, 0.5 ml of complement containing 2 units, and 0.25 ml of nuclei suspension or DNA solution were mixed and incubated in a waterbath at 37°C for 30 minutes or at 4°C overnight. One-half ml of sensitized cells was then added and a second incubation at 37°C for 30 minutes carried out. Serum, antigen, complement and cell controls were included in each test. Fixation of complement was read as complete if there was no

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TABLE I. Complement Fixation with Calf Thymus Nuclei.*

Serum	L.E. cell formation	Serum dilution 1	2	4	8	16	32	64	128	256	512
Syst. lupus eryth. (Fr.)	Strong	4	4	4	4	4	4	4	4	3	0
Idem (St.)	"	4	4	4	4	4	3	0	0	0	0
" (Ne.)	"	4	4	4	4	4	4	4	±	0	0
" (Ga.)	Medium	4	4	4	4	4	0	0	0	0	0
" (Hi.)	"	4	4	4	4	1	0	0	0	0	0
" (Ro.)	"	4	4	4	4	2	0	0	0	0	0
" (Sa.)	Weak‡	4	4	1	0	0	0	0	0	0	0
" (Ma.)	"	2	0	0	0	0	0	0	0	0	0
" (Ya.)	"	0	0	0	0	0	0	0	0	0	0
Misc. hyperglobulinemias	(9)	†	0	0	0	0	0	0	0	0	0
Rheumatoid arthritis	(21)	§	0	0	0	0	0	0	0	0	0
Miscellaneous diseases	(16)	†	0	0	0	0	0	0	0	0	0
Normal sera	(15)	†	0	0	0	0	0	0	0	0	0

* 2 mg/ml.

† A few of these sera were anticomplementary or showed slight fixation in the first 1 or 2 tubes, not considered significant.

‡ Those patients listed as "weak" gave distinctly positive preparations by the fresh heparin-bead method but weak to negative preparations by the Snapper method on stored sera.

§ Three of these sera had very high titers for sensitized sheep cell agglutinins. They were strongly anticomplementary. Parallel serum controls demonstrated that "fixation" in the first 2-3 tubes was due to the anticomplementary effect of the serum alone. The remaining 18 sera were negative throughout.

hemolysis (designated in Tables by 4), and as negative if there was complete hemolysis (designated in Tables by 0), with gradations of 3, 2, 1 and ±. A few sera were anticomplementary. In these instances parallel serum dilution controls to which no "antigen" was added were run for comparison, or the antigen dilution method employing a dilution of serum which was not anticomplementary was employed. *Absorption with nuclei and DNA*: Rabbit leukocyte or calf thymus nuclei were suspended in serum in a final concentration of 10 mg/ml, incubated in a waterbath at 37°C for 30 minutes, centrifuged at 2,000 rpm for 20 minutes and the supernate removed for testing. Lyophilized calf thymus DNA was added to serum in a final concentration of 1 mg/ml, incubated overnight at 37°C, centrifuged at 10,000 rpm for 2 hours, and the supernate removed for testing. In these experiments the L.E. test was done by the Snapper method.

Results. Experiments with cell nuclei: Positive complement fixation occurred with nuclei in 22 of 30 lupus sera. Results with representative lupus sera and a variety of other sera are presented in Table I. All lupus sera which were strong L.E. cell inducers showed complement fixation with nuclei at a dilution of 1:32 or greater. Lupus sera which were weak or negative L.E. cell inducers fixed complement in lower titer or failed to fix with calf thymus nuclei. The latter sera were usually from patients in clinical remission. Fixation was observed with calf thymus, human monocyte, beef liver and rabbit leukocyte nuclei. Most of the studies were carried out with calf thymus nuclei because of their availability.

The specificity of the reaction was tested on a limited scale by using sera from normal persons and from patients with other diseases characterized by hyperglobulinemia. Of the sera examined only those from patients with lupus erythematosus showed significant complement fixation. No significant reaction occurred with sera from 15 normal persons, 21 patients with rheumatoid arthritis, (3 of whom had very high titers of sensitized sheep cell agglutinins), 2 with Laennec's cirrhosis, 3 young patients with cirrhosis and marked hyperglobulinemia, 3 with macroglobulinemia, and 2 with multiple myeloma. In addition, single sera from patients with the following diseases were negative: hemochromatosis, Wilson's disease, infectious mononucleosis, unclassified collagen disease, subacute bacterial endocarditis, acquired hemolytic anemia

TABLE II. Complement Fixation by Representative L.E. Sera with Calf Thymus Nuclei* and Calf Thymus DNA.†

Serum	L.E. cell formation	Antigen	Serum dilution 1	2	4	8	16	32	64	128	256	512	1024
Fr.	Strong	Nuclei	4	4	4	4	4	4	4	4	2	0	0
		DNA	0	0	0	0	0	0	0	0	0	0	0
Ne.	"	Nuclei	4	4	4	4	4	4	1	±	0	0	0
		DNA	4	4	4	4	4	3	0	0	0	0	0
St.	"	Nuclei	4	4	4	4	4	3	0	0	0	0	0
		DNA	4	4	4	4	4	2	0	0	0	0	0
Ga.	Weak	Nuclei	4	4	4	4	3	0	0	0	0	0	0
		DNA	4	4	4	4	4	3	0	0	0	0	0
Hi.	"	Nuclei	4	4	4	4	0	0	0	0	0	0	0
		DNA	4	4	4	4	4	3	0	0	0	0	0

* 2 mg/ml suspension. † 100 γ/ml solution.

with cold agglutinins.

Experiments with DNA: Complement fixation also occurred with L.E. sera and calf thymus, salmon sperm, human leukocyte, and pneumococcal DNA. The degree of fixation with these substances was approximately the same. Most L.E. sera which showed fixation with nuclei also showed a reaction with DNA (Table II). However, serum Fr. was of particular interest because it was a strong L.E. cell inducer and fixed complement to high titer with nuclei, but showed no fixation with DNA. Two other sera with this behavior were encountered, but none was found which was capable of fixing complement with DNA but not with nuclei. Complement fixation with DNA was not observed in a control group of sera similar to those described above for the nuclei studies.‡

Absorption experiments: Cross-absorption with nuclei and DNA was carried out to determine whether the serum factors were identical. Absorption of L.E. serum with nuclei completely removed the ability of serum to induce L.E. cell formation and abolished or reduced complement fixation with nuclei. However, complement fixation with DNA was unaffected. On the other hand, after absorption of serum with DNA, complement fixation with DNA was significantly reduced but L.E. cell formation and fixation of complement with nuclei remained unimpaired (Table III).

TABLE III. Comparison of Effect of Absorption with Nuclei and DNA on Complement Fixation.

Serum	Absorbed with	"Antigen"	Serum dilution 1	2	4	8	16	32	64	128	256	512
Fr.	0	Nuclei*	4	4	4	4	4	4	4	4	1	0
	Nuclei	"	0	0	0	0	0	0	0	0	0	0
	DNA	"	4	4	4	4	4	4	4	4	4	1
	0	DNA†	0	0	0	0	0	0	0	0	0	0
	Nuclei	"	0	0	0	0	0	0	0	0	0	0
	DNA	"	0	0	0	0	0	0	0	0	0	0
Ne.	0	Nuclei*	4	4	4	4	4	4	4	±	0	0
	Nuclei	"	4	4	4	3	1	0	0	0	0	0
	DNA	"	4	4	4	4	4	4	1	0	0	0
	0	DNA†	4	4	4	4	4	4	2	±	0	0
	Nuclei	"	4	4	4	4	4	4	1	0	0	0
	DNA	"	4	2	0	0	0	0	0	0	0	0

* Calf thymus nuclei, 2 mg/ml. † Calf thymus DNA, 100 γ/ml.

‡ L. E. sera which fixed complement with nuclei also reacted strongly with calf thymus nuclear nucleoprotein. Of these sera, two also have fixed complement with histone in low titer.

Experiments with precipitin reaction: Considerable effort was directed toward demonstrating a specific precipitin reaction with DNA. Some of the lupus sera showed ring formation and a precipitate on adding DNA. However, the sera usually had considerable amounts of euglobulin that precipitated readily; the presence of DNA certainly facilitated the precipitation. Two control sera were encountered that also gave a precipitate with DNA. Both of these sera (1 from a patient with cirrhosis, the other from a patient with undiagnosed hyperglobulinemia) failed to give a positive complement fixation test with nuclei or DNA and did not produce L.E. cells. Among the lupus sera, however, a parallelism was noted between the precipitin reaction and complement fixation with DNA.

Discussion. A considerably larger group of patients with miscellaneous disorders will have to be studied before the specificity of the complement fixation with nuclei and with DNA can be clearly established. However, no positive reaction with either material was encountered in conditions other than lupus erythematosus in the present study. It will be of considerable interest to investigate serum from patients with hydralazine sensitivity and certain other disorders known to be associated with positive L.E. tests.

The evidence thus far obtained suggests a correlation between the L.E. test and complement fixation with nuclei. However, absolute comparisons were difficult: the complement fixation reaction is semi-quantitative and may react at high dilutions of L.E. serum while the L.E. phenomenon is difficult to quantitate and even the most active sera cannot be diluted very much without losing activity. In addition, many of the sera used had been stored for varying lengths of time and the ability of some of these sera to form L.E. cells could not be satisfactorily evaluated. Conclusions on this question will require simultaneous complement fixation and L.E. tests on fresh blood, using different methods for assaying L.E. cell formation.

Nuclei from a wide range of organs and species (rabbit leukocytes, calf thymus cells, beef liver cells and human monocytes) gave the complement fixation reaction. Although nuclear constituents probably were responsible for the reactions with these nuclei, a possible role of small cytoplasmic contaminants was not ruled out. The evidence suggested that human leukocyte and calf thymus nuclei were of comparable reactivity, and that this reaction compared favorably in sensitivity with the L.E. test.

The independent behavior of the complement fixation with DNA was unexpected. It had been the working hypothesis that cell nuclei absorbed the L.E. factor and fixed complement because of their content of nucleoprotein or DNA. The present data do not contradict this interpretation; however, the appearance of a separate complement fixing factor for DNA complicates the hypothesis. It is possible that both complement fixing factors are elicited by stimuli from nuclear material, the DNA factor being provoked by more dissociated fragments, and that the resultant factors have different specificities. However, the interrelationship between the serum factors remains unclear. In view of the results with the serum of patient Fr. and also the absorption experiments with other L.E. sera, it is unlikely that the separate DNA complement fixing factor plays a significant role in L.E. cell formation.

Reports of the production of DNA antibodies in experimental animals have appeared in the past(4,5). Other workers(6,7) have described complement fixation reactions with whole cell extracts in lupus erythematosus. These extracts probably contained nuclear materials which may in part have been responsible for the positive results, although cytoplasmic antigens undoubtedly played a role. The present observations suggest that antibodies to certain components of nuclei, including DNA, may be present in the serum of patients with lupus erythematosus. However, the exact nature of the reaction between the serum and nuclear material remains uncertain, and despite the fixation of complement, additional proof that this represents an antigen-antibody reaction is desirable.

Summary. 1. Sera from patients with active lupus erythematosus fixed complement with a wide variety of nuclei from different organs and species, with calf thymus nucleo-

protein, and in two instances with histone. Isolated calf thymus, salmon sperm, human leukocyte and pneumococcal DNA also fixed complement with many of these sera. Similar reactions were not encountered in a limited control series including normal individuals and other pathological states. 2. Most active L.E. sera fixed complement with both nuclei and DNA in roughly parallel titer. However, exceptions were encountered and one serum reacted strongly with nuclei but failed to react with DNA. Cross-absorption experiments with nuclei and DNA suggested the presence of 2 distinct serum factors. 3. The L.E. factor appeared to be related to the factor responsible for complement fixation with nuclei but distinct from that responsible for DNA fixation. 4. The significance of these findings with respect to antibodies against nuclear constituents is discussed.

Addendum: Recent reports by Miescher (*Vox Sanguinis,* 1957, v2, 283) and Seligmann (*Comptes Rendus,* 1957, v245, 1472) also have presented evidence for the presence of antibodies to DNA in lupus erythematosus serum.

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Beneficial Effect of Quinoxaline 1,4-di-N-Oxide in Radiation Injury in Mice.* (23546)

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One of the most prominent features of radiation syndrome in mice is a pronounced bacteremia which appears during the first post-irradiation week and materially contributes to the overall mortality. The type of organisms involved seems to vary with the particular colony of mice used but *Pseudomonas pyocyaneus, Proteus vulgaris, Salmonellae* and various coliform organisms are usually involved. Coulthard and Hale(1) recently showed that quinoxaline-1,4-di-N-oxide was effective in counteracting infection by these organisms in mice. Upon this basis the present investigation was undertaken and the results obtained indicate that, although the drug is not 100% effective, it does materially decrease irradiation bacteremia and total mortality in mice.

Methods. Four hundred male, CF 1 mice, weighing an average of 25 g, were arranged in groups of 20 animals each according to the design given in Table I. The quinoxaline-1, 4-di-N-oxide was administered intramuscularly or orally as an acacia suspension in normal saline. Control mice received similar doses of acacia in saline. Four additional groups of 32 animals each were used to determine the effects of the drug on the course of radiation bacteremia and mortality. Eight animals each from medicated and non-medicated groups were sacrificed on post-irradiation days 5, 8, 11 and 14 and the bacterial content of the liver was determined using direct dilutions on Tryptose (Difco) agar. Pure colonies were isolated and identified. The entire small intestine was similarly treated using

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