Reports

Affinity between the Lupus Erythematosus Serum Factor and Cell Nuclei and Nucleoprotein

The serum of many patients with systemic lupus erythematosus (L.E.) is capable of inducing characteristic alterations in white blood cells *in vitro*. The alterations include swelling of the nucleus of some white cells and the appearance of bodies resembling these swollen nuclei in the cytoplasm of other intact polymorphonuclear leucocytes. The latter cells are known as L.E. cells. How the serum induces these cellular changes has remained obscure. However, the observed morphological changes, the fact that the inclusion body of the L.E. cell stains with Feulgen reagent (*1*) and, in particular, the recent work of Miescher (*2*), strongly indicate a reaction specifically involving cell nuclei. This report presents evidence suggesting that the responsible serum factor combines directly with cell nuclei and nuclear nucleoprotein.

Starch-zone electrophoresis of highly active L.E. serum was carried out. It confirmed the finding of others (3) that the factor migrates with the faster portion of the γ-globulin. Ultracentrifugation of L.E. serum in a sucrose gradient and also in saline solution showed that the factor sediments with the bulk of the γ-globulin with an *s* rate of approximately 7*S*.

Nuclei from calf thymocytes, rabbit polymorphonuclear leucocytes, and human monocytes were prepared in sucrose-calcium solution, sometimes containing citric acid. When each of these types of nuclei was incubated for 30 minutes in highly positive L.E. sera at temperatures between 18° and 38°C and the nuclei then removed by centrifugation, the sera lost completely their ability to induce L.E. cell formation. Electrophoretic and immunological determination of total serum γ-globulin before and after absorption with nuclei showed little change. In one case, the γ-globulin was 20.5 mg/ml before and 19.8 following absorption.

Adherence of the serum factor to the nuclei is suggested by the fact that nuclei which had been removed from L.E. serum, washed with cold saline until the solution was free of protein, and then incubated with fresh human white blood cells, were readily phagocytized to form L.E. cells. Nuclei exposed to normal serum and treated in an identical manner were not similarly phagocytized.

The absorbed L.E. factor could be removed partially from nuclei by incubating the nuclei in isotonic saline for 20 minutes at temperatures from 45° to 65°C. The eluted protein, which was active, was found to be γ-globulin and was quantitatively precipitated by antiserum to normal γ-globulin. The nuclei after elution were still phagocytized to form L.E. cells, indicating that the elution had been incomplete.

In an effort to determine the reactive component of the nuclei, similar experiments were conducted with isolated nuclear nucleoprotein. Nucleoprotein was extracted in 1*M* NaCl. A crystal-clear, viscous solution was obtained from which strands of nucleoprotein could be precipitated by dilution to physiological salt concentration. After absorption with the nucleoprotein, L.E. serum was unable to induce L.E. cell formation. When the nucleoprotein, after incubation with L.E. serum, was washed and incubated with fresh white blood cells, strands of nucleoprotein were phagocytized by the white blood cells to form inclusion bodies very similar to those of the L.E. cells. Nucleoprotein incubated with normal serum or saline was not so phagocytized.

Following treatment with deoxyribonuclease sufficient to remove approximately 50 percent of the deoxyribonucleic acid the nuclei would absorb some of the L.E. factor and would readily release all of that absorbed on subsequent incubation at 56°C. If all the deoxyribonucleic acid was removed with deoxyribonuclease, none of the factor could be absorbed. On the other hand, treatment with ribonuclease did not impair absorptive capacity. After selective removal of the histone by extraction of the nuclei with 0.5-percent citric acid in 1*M* NaCl, the ability of the nuclei to absorb factor remained, but was somewhat diminished.

Exposure of nuclei to normal serum, to cirrhotic serum with very high γ-globulin, and to serum with large amounts of euglobulin did not make the nuclei susceptible to phagocytosis by white cells with formation of L.E. cells, nor did it interfere with their ability to absorb the L.E. factor. However, absorption of factor was prevented by prior treatment of nuclei with protamine, which is thought to bind the phosphate groups of deoxyribonucleic acid, and with atabrine. The latter drug appears to have a therapeutic effect in lupus erythematosus.

Further exploration of the reaction between L.E. serum and cell nuclei was accomplished by studying the L.E. cell reaction by the fluorescent antibody technique during the course of other studies conducted in cooperation with Robert Mellors and Louis Ortega of the Sloan-Kettering Institute (*4*). Slides containing L.E. cells were reacted with fluorescent rabbit antiserum to normal human γ-globulin. Nuclei which were undergoing change preliminary to phagocytosis during L.E. cell formation (Fig. 1) and the inclusion bodies of L.E. cells fluoresced brilliantly. There was no similar fluorescence of the nucleus of the phagocytic cell, or of any nuclei in control preparations made with normal serum. Thus the localization of γ-globulin, presumably L.E. factor, on the affected nuclei during *in vitro* L.E. cell formation could be demonstrated.

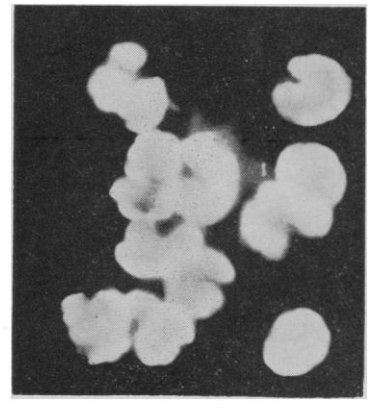

Fig. 1. Visualization of L.E. cell preparation made with L.E. serum and stained with fluorescent antibody to human γ-globulin. Marked fluorescence of swollen white cell nuclei is visible. In identical preparations made with normal serum as controls, there was no nuclear fluorescence.

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These data suggest that the L.E. serum factor has an affinity for nuclear nucleoprotein and that deoxyribonucleic acid is involved in the bond. The fact that the L.E. serum factor is a γ-globulin that appears to react with antiserum to normal γ-globulin suggests that the factor may be an antibody. This possibility merits further investigation, with particular reference to the question of whether or not the L.E. factor could be an autoantibody to nucleoprotein or deoxyribonucleic acid.

Note added in proof: Recently we have learned that George Friou of West Haven, Conn., has applied the fluorescent antibody technique to the study of properties of L.E. serum and has obtained results similar to those reported here.

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References

1. M. M. Hargraves, H. Richmond, R. Morton, *Proc. Staff Meetings Mayo Clinic* **23**, 25 (1948).
2. P. Miescher and M. Fauconnet, *Experientia* 10, 252 (1954).
3. J. R. Haserick, L. A. Lewis, D. W. Bortz, *Am. J. Med. Sci.* **219**, 660 (1950).
4. R. C. Mellors, L. G. Ortega, H. R. Holman, *J. Exptl. Med.*, in press.

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Interface Enrichment of Methylene Blue by Fatty Acids with Microanalytic Applications

One of us (G.M.) has observed that thionine dyes are attracted by fatty acids to the interface between alkaline water and water-immiscible fatty-acid solvents. This attraction provides the basis for a convenient new method of measuring very small amounts of certain long-chain fatty acids. In particular, palmitic, oleic, stearic, elaidic, and linoleic acids, separately or in mixtures, have been shown to cause the basic dye, methylene blue, to become enriched at the interface of a two-phase system consisting of a heptane mixture (Skellysolve D) plus methylene blue in ammonia water. The loss of dye from the aqueous bulk phase is a logarithmic function of the amount of such fatty acids in the system. Thus, total concentration of the afore-mentioned fatty acids may be determined by application of this method.

To determine the fatty acids, a solution of 500 μg of methylene blue chloride (minimum assay, 98.5 percent) in 100 ml of 0.6*M* NH_4OH was prepared within an hour before use. Fatty acid working standard solutions containing 100 mμ mole of reagent-grade acid per milliliter of Skellysolve D solution were prepared and kept in glass-stoppered bottles (stoppers were wetted with glycerol to prevent evaporation) away from light. Standard curves for the fatty acids usually were made with mixtures simulating natural fats. Final volumes of 2 ml for the hydrocarbon phase were used in the determinations. Typical standard curves were made by pipetting 0, 20, 40, 80, 120, and 160 millimicroequivalents of the acids into sulfuric acid-dichromate cleaned Coleman spectrophotometer cuvettes (19 by 105 mm round). Into each tube, 6-ml volumes of the ammonia-methylene blue solution were added. All tubes were capped with Saran wrap covered stoppers and shaken 100 times vigorously in a rack. Immediately after this, the cuvettes were placed in a size 2, model V International centrifuge, and the power was adjusted to bring the centrifuge up to 1600 rev/min in 60 seconds; the centrifuge was then slowly braked to a stop. The cuvettes were carefully removed to prevent disturbance of the interface. The optical density was read immediately in a Coleman universal spectrophotometer at 665 mμ. The running of a standard curve with each set of unknowns, and uniform standardization of the procedure, and the maintenance of a constant ambient temperature were found to be very critical factors because this system is not in equilibrium when readings are made.

Table 1 shows typical optical density values observed. These were obtained using a mixture of fatty acids similar to lard (*1*). The precision of the standard curve is indicated by the function showing percentage standard error of duplicate analyses. This function was obtained from the formula

$$\frac{\sqrt{\dfrac{\Sigma(x_1 - x_2)^2}{2}}}{\dfrac{n-1}{\bar{x}}} \times 100 = \text{percentage standard error of duplicate analyses.}$$

In this formula,

$$\frac{x_1 - x_2}{2}$$

is the range in millimicroequivalents around the mean of duplicate values, $\bar{x}$; the number of sets of duplicates is denoted by n.

Preliminary studies have shown that this procedure can be used for the measurement of unesterified long-chain fatty acids in plasma (0.2 to 0.5 ml of sample is needed) and of total saturated and unsaturated long-chain fatty acids after alkaline hydrolysis of a 0.1-ml sample.

Furthermore, a check of this method was made on ethanolic KOH saponified samples of lard and sunflower seed oil. The amount of fatty acids found by titration of 4-g fat samples checked within 3 percent of the values obtained using the methylene blue interface enrichment method on aliquots of the lard and sunflower seed oil soaps.

Table 1. Typical optical density values obtained in the determination of total long-chain fatty acids with percentage standard error for duplicate readings. The mixture contained palmitic, stearic, oleic, and linoleic acids in the molar ratios of 5/3/9/1.

Total fatty acid (mμmole)	Optical density	Percentage of standard error for duplicate readings
10	1.030	18.3
20	0.960	4.6
40	0.810	3.6
80	0.525	3.1
120	0.320	6.6
160	0.245	11.2
200	0.220	8.4

It should be stated that caprylic and lauric acids do not cause any observable concentrations of methylene blue at the interface. Moreover, linolenic acid and myristic acids only weakly retain methylene blue at the interface in these procedures. However, iodinated linolenic acid behaves in the same manner as palmitic, oleic, stearic, elaidic, and linoleic acids. Nevertheless, it is evident that this method may be applied for assay of nearly all the long-chain fatty acids in mammalian blood and tissue lipids and in vegetable lipids, except butter and some vegetable fats.

This new method is not only more sensitive but also more practicable than previously available methods for measuring long-chain fatty acids: the materials and equipment required are within the scope of any basically equipped chemistry laboratory.

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Reference

1. T. P. Hilditch, *The Chemical Composition of Natural Fats* (Wiley, New York, 1947).

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Successive Reversal of a Position Habit in an Invertebrate

The performance of white rats on a series of discrimination reversals based on spatial cues (*1*) has been extensively investigated. These studies show that after a very brief period of negative transfer there occurs a gradual reduction in the number of errors with each succeeding reversal. This interreversal im-