# THE REACTION OF THE LUPUS ERYTHEMATOSUS (L.E.) CELL FACTOR WITH DEOXYRIBONUCLEOPROTEIN OF THE CELL NUCLEUS

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(Submitted for publication May 25, 1959; accepted July 10, 1959)

Previous reports have presented evidence that the lupus erythematosus (L.E.) cell factor reacts with deoxyribonucleoprotein of the cell nucleus, and that deoxyribonucleic acid (DNA) is essential for the reaction (1, 2). More recently, a number of other serum factors have been identified in systemic lupus erythematosus (SLE) which react with other constituents of the nucleus. These include isolated DNA, purified histone and a material extractable from the nucleus with isotonic buffers which is neither nucleic acid nor histone (3, 4).

These reactions with nuclear constituents have been demonstrated by standard immunological techniques such as complement fixation (5, 6), precipitation (7, 8), hemagglutination (9), latex agglutination (10) and passive cutaneous anaphylaxis (11). The serum factors, including the L.E. cell factor, migrate with γ-globulin on electrophoresis and sediment with γ-globulin on centrifugation. These results suggest that the L.E. cell factor is but one of a group of antibodies to different components of the nucleus which arise in SLE. However, knowledge of the precise reactivities of the antinuclear factors is necessary in order to establish that they are antibodies and to permit study of their origin and biologic roles. The L.E. cell factor provides an example of the need for such information. Already three different nuclear constituents have been suggested as the substance with which this factor reacts: intact deoxyribonucleoprotein (1, 2, 12), DNA alone (13) and a protein of the nucleus but not DNA (14).

If the L.E. cell factor is an antibody, it should exhibit a particular antigenic specificity and, when isolated, should be indistinguishable from typical human antibody γ-globulin. The work reported in this paper comprises an effort to identify the nuclear component upon which the L.E. cell factor acts, and to isolate the factor and define certain of its properties. The term L.E. cell factor is used to signify only that factor which induces formation of L.E. cells, and none of the other antinuclear factors.

Because fractionation of the cell nucleus is still in a primitive stage and because many of the methods employed in this study have not been extensively used and verified, the experimental details will be given together with the results.

## MATERIALS AND GENERAL METHODS

Sera were collected and stored under sterile conditions at 4° C., usually with 1 : 10,000 concentrations of merthiolate.

Nuclei were obtained from calf thymus glands at 4° C. by the method of Mirsky and Pollister (15). Glands were minced with scissors, homogenized in a solution of 0.25 M sucrose and 0.003 M $CaCl_2$ in a Waring blendor at a speed which did not disrupt the nuclei, filtered through gauze and flannel and the nuclei separated by centrifugation. The resulting nuclei were intact microscopically and had minimal cytoplasmic contamination. For storage, nuclei were lyophilized.

Nucleoprotein was prepared from unlyophilized nuclei by extraction overnight at 4° C. with 1 N NaCl (16). The resulting viscous suspension was centrifuged at 78,000 × G for 90 minutes in the Spinco "L" ultracentrifuge and the clear viscous supernate solution of the nucleoprotein stored at 4° C. Such solutions remained stable for at least 30 days under these conditions. Nucleoprotein was obtained for experimental use by diluting the stock solution with six volumes of cold distilled water, thus bringing it to physiologic salt concentration. Silvery nucleoprotein strands precipitated. These strands were separated by centrifugation and homogenized in a Teflon tissue grinder in isotonic saline. Equal volumes of the homogenate were then removed, spun, and pellets of solid, particulate nucleoprotein used for absorption. In general, nucleoprotein pellets containing 1 to 1.5 mg. DNA were used to absorb 0.5 ml. of serum, although pellets half that size are adequate to absorb completely 0.5 ml. of active serum if an incubation time of 45 minutes at 37° C. were allowed.

Human white cell nuclei were prepared from leukocytes from patients with granulocytic and monocytic leukemia. The above methods were used except that the cells were disrupted in a Teflon tissue grinder in a solution of 0.25

TABLE I

*Typical examples of absorption of protein from systemic lupus erythematosus (SLE) serum by nucleoprotein**

| | | Material absorbed | | | | | |
|---|---|---|---|---|---|---|---|
| | | Saline | Normal serum | Hyper-γ-glob. serum | SLE serum | SLE serum | SLE serum |
| L.E. prep. with absorbed serum | | | | | 0 | 0 | ± |
| Analysis of pellet after absorption | Total DNA† *mg.* | 1.00 | 0.81 | 0.81 | 0.92 | 1.00 | 0.94 |
| | Total protein *mg.* | 0.90 | 0.70 | 0.70 | 1.23 | 1.56 | 1.17 |
| | Protein/DNA† ratio | 0.90 | 0.86 | 0.86 | 1.33 | 1.56 | 1.24 |

* Equal aliquots of serum or saline were absorbed with pellets of nucleoprotein for 45 minutes at 37° C. Pellets were then washed and total DNA and protein determined. Data are expressed in terms of pellets containing 1 mg. DNA at start of experiment.

† DNA = deoxyribonucleic acid.

M sucrose, 0.003 M $CaCl_2$ and 0.2 per cent citric acid. Human leukocyte nucleoprotein was obtained from unlyophilized nuclei in the manner employed for calf thymus nucleoprotein.

Calf thymus DNA was purchased from the Mann Research Laboratories, Inc., New York, and showed the following analyses: C, 37.18 per cent; N, 16.54 per cent; P, 9.91 per cent. DNA concentrations were determined by absorption at 260 mμ in a Beckman Spectrophotometer. The material to be analyzed was heated to 70° C. for 30 minutes in 10 per cent perchloric acid, centrifuged and the clear supernate used for assay.

Protein concentrations were determined by the modified method of Folin-Ciocalteau (17). Solid materials were dissolved in 0.1 N NaOH prior to removal of aliquots for protein determination.

Calf thymus histone prepared by acid extraction of nucleoprotein was purchased from Worthington Chemical Corporation, Freehold, New Jersey. Lysine-rich histone prepared from calf thymocytes was kindly donated by Dr. Alfred Mirsky of the Rockefeller Institute. Standard curves for determining histone concentration were constructed using solutions of these histones and the Folin-Ciocalteau method.

Once crystallized pancreatic deoxyribonuclease (DNase) was obtained from Worthington Chemical Corporation. All experiments employing this enzyme were done with fresh solutions of enzyme in phosphate buffer, ionic strength 0.1, pH 6.8, and containing 1 μMole of magnesium for every 0.1 mg. of DNase.

L.E. cell preparations were made by the method of Snapper and Nathan (18) using a mixture of the material to be tested and fresh normal human blood. This method permits localization of the L.E. cells in a small area which facilitates reading and comparing of slides. Only sera which formed large numbers of L.E. cells were studied. Removal or inhibition of the L.E. cell factor in these sera resulted in a striking reduction or complete disappearance of L.E. cell formation which could be readily identified. Thus the results could be reported in most instances as highly positive (++++), negative (0), or very weakly positive (±). No satisfactory method for quantification of L.E. cell formation exists.

Complement fixation was studied by the method of Casals and Palacios (19) as applied to this system (5). Two 100 per cent units of complement were used and visual endpoints recorded with appropriate serum and antigen controls. When materials containing DNase were used for complement fixation, sodium citrate was added in excess of the magnesium concentration and all reagents employed were free of both calcium and magnesium.

Precipitin reactions in agar plates were conducted by the method of Ouchterlony (20) employing 0.5 per cent agar in isotonic NaCl or appropriate buffer. Double diffusion precipitin reactions in agar were conducted as outlined previously (8).

Fluorescent antibody studies were conducted with Drs. Mellors and Ortega utilizing techniques previously described (21).

Rabbit antisera to Red Cross or Lederle Fraction II human γ-globulin, Red Cross human albumin and whole normal human serum were employed for immunologic assays. Antiserum to 19S human γ-globulin was prepared by absorbing rabbit antiserum to normal human γ-globulin (separated by electrophoresis) with Fraction II γ-globulin, or by immunizing rabbits with isolated human macroglobulins. Estimations of γ-globulin were made by the capillary tube precipitation method, with gradation of the interface precipitate from trace to (++++) amounts. Quantitative determinations of γ-globulin were made by the standard precipitin method, using 0.5 ml. of the appropriate dilutions of γ-globulin solution, 0.5 ml. buffer and 0.05 ml. of the standardized antiserum.

## EXPERIMENTS AND RESULTS

### *Adsorption of γ-globulin and L.E. cell factor by nucleoprotein*

Examples of the highly specific absorption of protein from SLE serum by nucleoprotein are

seen in Table I. Equal amounts of homogenized calf thymus nucleoprotein were used to absorb equal volumes of isotonic saline, normal human sera, sera from patients with hypergammaglobulinemia due to liver disease, and SLE sera. After absorption, the nucleoprotein was washed until the wash fluid was free of protein and the pellets were then analyzed for total protein and total DNA. The ratio of protein to DNA remained about the same in that nucleoprotein which was used to absorb saline, normal serum, and serum with hypergammaglobulinemia. However, this ratio was increased greatly in the nucleoprotein pellets used to absorb SLE serum. Ratios greater than 0.91, which was that of unaltered nucleoprotein, were found only after exposure to SLE serum. The total amounts of protein and DNA were reduced in the pellets used to absorb normal sera and hypergammaglobulinemic sera, though the ratio remained the same. This was due to dissolving of nucleoprotein during absorption. It is possible that a protective effect of the adherent γ-globulin prevented a similar degree of solution in the SLE serum.

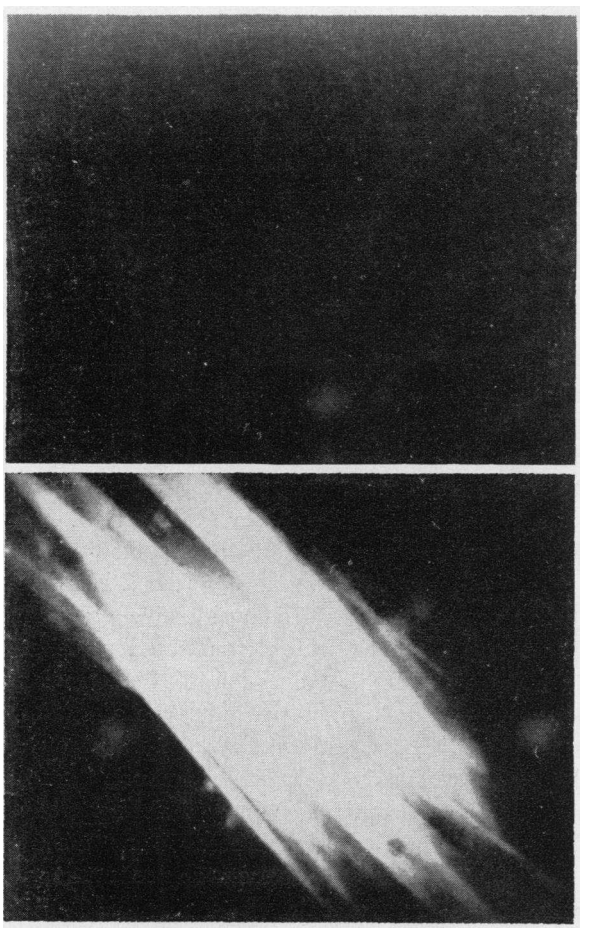

FIG. 1. ABSORPTION OF γ-GLOBULIN BY NUCLEOPROTEIN EXPOSED TO SLE SERUM

Identification of γ-globulin on nucleoprotein by fluorescent antibody method. Upper frame is photograph of nucleoprotein strands exposed to normal serum, washed and stained with fluorescein-labeled rabbit antibody to normal human γ-globulin. Lower frame is photograph of nucleoprotein strands exposed to SLE serum and treated similarly.

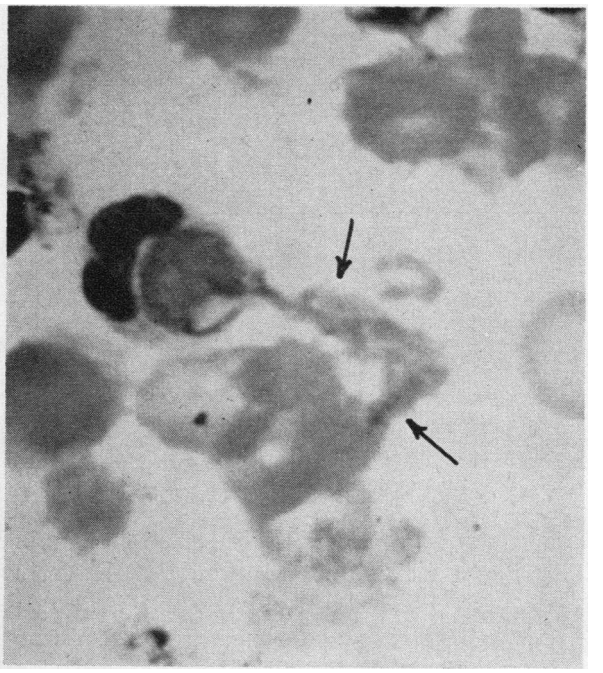

FIG. 2. PHAGOCYTOSIS OF A NUCLEOPROTEIN STRAND COATED WITH L.E. CELL FACTOR

Strands of nucleoprotein were used to absorb SLE serum, washed and incubated at 37° C. for one hour with fresh, normal human leukocytes in normal plasma. Smears were then made. Arrows indicate nucleoprotein strand being ingested by the leukocyte at the left. Many erythrocytes are present. After staining with Wright's stain, the nucleoprotein was light blue and the cell nuclei were dark blue.

The absorption of γ-globulin from SLE serum by nucleoprotein serum may be seen in Figure 1. In this experiment, strands of nucleoprotein were used to absorb both normal and SLE serum. The nucleoprotein strands were then washed six times and stained with antibody to normal human γ-globulin labeled with fluorescein. In the upper black frame nucleoprotein exposed to normal serum was stained, and none of the fluorescent

TABLE II

*Recovery of lupus erythematosus (L.E.) cell factor from complex with nucleoprotein**

| 1 | 2<br>56°<br>Eluate<br>from<br>com-<br>plex | 3<br>Supernate<br>from<br>DNase<br>digestion<br>of complex | 4<br>56°<br>Eluate<br>from<br>digestion<br>residue | 5<br>Residue<br>from<br>56°<br>elution in<br>Column 4 | 6<br>2N NaCl<br>37° C.<br>eluate from<br>digestion<br>residue | 7<br>Residue<br>from<br>2N NaCl<br>elution in<br>Column 6 |
|---|---|---|---|---|---|---|
| % Histone | ±2 | 15–20 | 5–10 | 75–85 | | |
| % DNA | ±2 | 75–85 | 5–10 | 10–20 | | |
| γ-Globulin | ± | ++ | +++ | present | +++ | present |
| L.E. cell factor | ± | ± | ++++ | | ++++ | |
| Complement fixation | 0 | DNA, nuclei, nucleoprotein | nuclei, nucleoprotein | | | |
| Precipitation with DNA | 0 | 0 | 0 | | | |

* Complexes were made by incubating pellets of nucleoprotein containing 1.3 mg. of deoxyribonucleic acid (DNA) with 0.5 ml. of serum. Liberated and residual DNA and histone were determined on identical aliquots of nucleoprotein run in parallel but incubated only with saline. Per cent DNA and per cent histone refer to per cent of total in original pellet. For further details, see text.

antiglobulin was bound. The nucleoprotein in the lower frame was passed through SLE serum and is seen to be brilliantly fluorescent, indicating that it had absorbed γ-globulin from the serum which then reacted with the fluorescent antibody.

After absorption with nucleoprotein, SLE serum was no longer able to induce L.E. cell formation, indicating specific removal of the L.E. cell factor. The nucleoprotein from this absorption became susceptible to phagocytosis by viable white cells with the formation of inclusion bodies very similar to the inclusion bodies of typical L.E. cells (Figure 2). The inclusion bodies appeared homogeneous and stained light purple with Wright's stain. No such phagocytosis occurred with nucleoprotein exposed to normal serum.

*Recovery of L.E. cell factor from the complex with nucleoprotein*

Table II summarizes these experiments. Column 1 lists the assays made on solutions obtained during efforts to recover the factor from nucleoprotein after absorption of SLE serum; the other columns give the results of these assays after the steps indicated at the top of the columns. Column 2 shows that elution at 56° C. in isotonic saline did not liberate significant amounts of the constituents of nucleoprotein, of γ-globulin, or of L.E. cell factor. Column 3 indicates that after digestion of the nucleoprotein complex with deoxyribonuclease, a small amount of the histone, a large amount of the original DNA and considerable γ-globulin were recovered. This γ-globulin had little if any capacity to induce formation of L.E. cells but was capable of fixing complement with isolated DNA, isolated nuclei and nucleoprotein.

If the residue remaining after DNase digestion was eluted at 56° C. in isotonic saline, an additional large amount of γ-globulin was recovered which possessed great ability to induce L.E. cell formation. This γ-globulin was capable of fixing complement with nuclei and nucleoprotein but not with isolated DNA (Column 4). The residue which remained after both DNase digestion and elution at 56° C. contained most of the original histone, a small amount of the original DNA and a certain amount of γ-globulin (Column 5). An alternative procedure to the elution at 56° C. in isotonic saline was elution at 37° C. in 2 N saline. This procedure also yielded considerable γ-globulin and much L.E. cell factor from the residue remaining after DNase digestion of the complex. Columns 6 and 7 summarize this type of elution.

Identical results were obtained with each of 20 SLE sera studied by this method, despite the fact that these sera contained widely differing groups of other antinuclear antibodies. Thus the recovery of L.E. cell factor from nucleoprotein required first digestion of the DNA by DNase and then elution of the factor from the residual material, which is primarily histone and nonhistone protein. This sequence of procedures was therefore used as a standard method throughout subsequent experiments designed to study the reaction of the L.E. cell factor with nucleoprotein.

A number of the techniques used in the experiments in Table II require further explanation. *1*)

The attachment of L.E. cell factor to the residue after DNase digestion is weakened by heat. Not only is the factor liberated at 56° C., but small amounts of it are liberated at room temperature and below. Therefore the digestions with DNase were conducted at 4° C. for 15 to 18 hours to minimize elution of L.E. cell factor from the digestion residue during the course of the digestion. Judged by removal of DNA, digestion at 4° C. for 15 hours is as effective as digestion at 37° C. for one hour. The DNA remaining after this digestion appears to be an "indigestible" residue; further exposure to DNase does not lead to further removal of DNA. *2*) Assay of the DNase supernate for L.E. cell formation proved difficult initially. The DNase present in the supernate destroyed the nuclei of the dead "substrate" leukocytes during L.E. cell tests and thus prevented formation of L.E. cells. However, the enzyme was inactivated by heating to 65° C. for 45 minutes while the L.E. cell factor was not. Therefore, DNase supernates were heated to 65° C. for 45 minutes prior to assay of their ability to form L.E. cells. Such heated supernates were shown to be both incapable of inhibiting L.E. cell formation when mixed with solutions of L.E. cell factor and incapable by themselves of causing more than occasional, minimal formation of L.E. cells. This latter activity appeared to be little more than would dissociate if the residue from enzyme digestion were allowed to stand overnight at 4° C. in the absence of enzyme. *3*) The γ-globulin still adherent to the residue of the complex after both DNase treatment and 56° C. elution was identified by a modified γ-globulin consumption method. The insoluble residue was divided into equal aliquots; one was incubated with normal rabbit serum and the other with rabbit antiserum to human γ-globulin for one hour at 22° C. and overnight at 4° C. The tubes were then spun and the pellets washed twice. Total protein was determined on each pellet; much more was present in the pellet incubated with antiserum. This indicates adsorption of antiglobulin protein and therefore also the presence of γ-globulin on the residue. This γ-globulin could not be removed from the residue by further digestion with DNase, further elution at 56° C. or elution in 2 N NaCl. It therefore has not been characterized.

Evidence obtained with the two step recovery technique underscored the specificity of the adsorption of γ-globulin from SLE serum by nucleoprotein. If normal serum was absorbed with nucleoprotein, which was then digested with DNase and the residue eluted at 56° C. with isotonic saline, no γ-globulin was recovered. If the same was done with sera from other diseases containing levels of γ-globulin much higher than those present in SLE sera, only a trace or no γ-globulin was recovered.

*Identification of constituents of nucleoprotein which react with the L.E. cell factor*

In these and subsequent experiments, five sera were used which contained large amounts of L.E. cell factor and varying types of other antinuclear factors. The results obtained were the same for the five sera.

Prior to absorption of SLE serum, nucleoprotein was digested with DNase to remove the DNA, or extracted with 0.1 N HCl to remove histone. Both procedures left insoluble residues which were then employed to absorb the serum. The amounts of nucleoprotein, present at the starting point, and of serum used for absorption were kept

TABLE III

*Effect of removal of deoxyribonucleic acid (DNA) or histone on ability of nucleoprotein to absorb L.E. cell factor**

| | Untreated control nucleoprotein | Nucleoprotein with DNA removed | Nucleoprotein with histone removed |
|---|---|---|---|
| DNA before absorption (*mg.* total in pellet) | 1.32 | 0.19 | 1.32 |
| Protein before absorption (*mg.* total in pellet) | 1.10 | 0.83 | 0.20 |
| L.E. cell formation absorbed serum | 0 | ++++ | ++++ |
| DNA after absorption (*mg.* total in pellet) | 1.32 | | 1.28 |
| Protein after absorption (*mg.* total in pellet) | 1.70 | 0.64 | |
| L.E. cell formation DNase supernate | ± | 0 | 0 |
| L.E. cell formation 56° supernate | ++++ | + | + |

* The nucleoprotein pellet was digested at 37° C. for one hour in 0.5 ml. of a solution of 0.2 mg. DNase per ml. of buffer, or was extracted at room temperature in 1 ml. of 0.1 N HCl for 15 minutes. The residues were washed three times before use for absorption.

constant. Results are seen in Table III. In the column labeled "Untreated control nucleoprotein," the results of a typical absorption with untreated nucleoprotein are given. The serum was unable to induce L.E. cell formation after absorption and highly active L.E. cell factor could be recovered from the nucleoprotein. However, nucleoprotein from which DNA or histone was largely removed was able to absorb only very small amounts of L.E. cell factor. In the experiment in which histone was removed, the amount of DNA present after the attempted absorption was the same as that present initially, demonstrating that DNA had not dissolved during the absorption. The histone residue after removal of DNA dissolved partially on absorption. However, this experiment was done repeatedly using larger amounts of histone residue with the same volume of serum, and only very slight absorption of L.E. cell factor occurred.

By the methods employed, it was never possible to remove from the nucleoprotein all of the DNA by digestion or all of the protein by extraction. The absorption of small amounts of L.E. cell factor by these altered nucleoproteins was presumably due to a small residual amount of nucleoprotein which resisted enzyme digestion or acid extraction.

Because nucleoprotein from which either DNA or histone had been removed had a greatly reduced capacity to absorb L.E. cell factor, an effort was made to recombine the separated portions and to determine whether or not a "reconstituted" nucleoprotein regained its reactive capacity. After removal of DNA with DNase, efforts at reconstitution by incubation of the washed residue with a solution of purified DNA equal in amount to that removed were unsuccessful. Little DNA combined with the residue and the product did not differ significantly from the residue before recombination. Reconstitution was achieved, however, after extraction of histone from the nucleoprotein by acid. In these experiments nucleoprotein pellets containing 1.3 mg. of DNA were extracted for 15 minutes at room temperature with 1 ml. of 0.1 N HCl. This removed about 90 per cent of the protein. The extract was then dialyzed overnight against isotonic NaCl to remove acid and the next day the extracted protein was recombined with the residue in a homogenizer at room temperature. Residue and extracted protein recombined quantitatively yielding a product almost identical in composition to the original nucleoprotein. This reconstituted nucleoprotein gave evidence of some return of reactive capacity. It was capable of absorbing some L.E. cell factor from serum, and $\gamma$-globulin and L.E. cell factor could be recovered from the reconstituted nucleoprotein after absorption. However, under the conditions we have employed we have not succeeded in obtaining return of more than about 50 per cent of the original reactivity; when compared with normal nucleoprotein on a weight basis, twice as much reconstituted nucleoprotein was necessary to absorb completely a given amount of serum.

Next, an effort was made to determine whether other long chain acids or basic proteins could be substituted for the DNA or histone of the nucleoprotein. These results with "synthetic" complexes are summarized in Table IV. The "synthetic" nucleoprotein which is obtained from a mixture of purified DNA and isolated histone possesses some capacity to absorb L.E. cell factor, but not the capacity of an equivalent amount of control nucleoprotein. Pellets of this "synthetic" nucleoprotein contained slightly more DNA than the control nucleoprotein. However, it proved extremely difficult to duplicate in the "synthetic" DNA-histone the exact protein to DNA ratio found in extracted nucleoprotein. The latter ratio was consistently 0.91 by our methods of assay. Despite repeated experiments with wide variation in the amounts of histone and DNA mixed, the resulting "synthetic" nucleoprotein possessed a ratio close to 1.0, indicating approximately 10 per cent more histone per unit of DNA than in extracted nucleoprotein. Substitution of protamine for histone in the reaction with purified DNA gave a product with the appearance of extracted nucleoprotein but completely devoid of reactivity. In this case weight-combining ratios of protamine to DNA near 0.5 were used because the molecular weight of protamine is estimated to be about one-third that of histone. It was assumed that with such a ratio, the saturation of binding sites on the DNA with protamine would more closely approximate the saturation of DNA with histone in control nucleoprotein. Here too, we could not achieve a ratio lower than 0.5. Substitution of heparin, hyaluronic

TABLE IV

*Absorption of L.E. cell factor by "synthetic" nucleoprotein, long chain acid complexes with histone and protamine, and DNA alone**

| | | Control nucleo-protein | Synthetic DNA-histone | Synthetic DNA-protamine | Heparin-histone | Heparin-protamine | Hyaluronic acid-histone | Poly-ethylene sulfonate-histone | DNA alone |
|---|---|---|---|---|---|---|---|---|---|
| L.E. prep. with abs. serum | | 0 | ++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Supernate DNase digestion | γ-Globulin | ++ | ++ | + | | | ft. tr. | 0 | ++ |
| | L.E. prep. | ± | + | 0 | | | | | ± |
| Supernate 56° elution | γ-Globulin | ++ | + | tr. | tr. | 0 | 0 | 0 | 0 |
| | L.E. prep. | ++++ | ++ | 0 | | 0 | 0 | | 0 |

* The synthetic substances were made by mixing saline solutions of the components at room temperature, whereupon precipitation occurred. The control nucleoprotein contained 1.2 mg. DNA with a protein:DNA ratio of 0.91. The DNA-histone contained 1.4 to 1.6 mg. DNA with a protein:DNA ratio of near 1.0. The DNA-protamine contained up to 2 mg. DNA with a protein:DNA ratio near 0.5. The heparin-histone, heparin-protamine, hyaluronic acid-histone and polyethylene sulfonate-histone precipitates were obtained by mixing solutions containing 2 mg. of each reactant. The precipitates contained 1 to 1.5 mg. of the protein. All precipitates were used to absorb 0.5 ml. aliquots of the same L.E. serum. 1.2 mg. pure DNA was used for absorption.

acid or polyethylene sulfonate for DNA in the reaction with histone also yielded solid complexes which could be used for absorption but which were inactive. Thus it appears that not only are both the DNA and the histone of the nucleoprotein necessary for the reaction with L.E. cell factor but also that the specific combining configuration present in the nucleoprotein extracted from nuclei is most efficient, and was apparently not obtained in the various reconstituted or "synthetic" nucleoprotein preparations.

For purposes of comparison, data resulting from attempts to absorb L.E. cell factor with highly purified DNA are given in the last column of Table IV. This experiment was done in two ways. First, DNA in amounts equal to that normally present in the standard pellet of homogenized nucleoprotein was added to SLE serum and allowed to incubate for 30 minutes at 37° C. Minimal agitation was used so that the DNA did not dissolve, but simply formed a gelatinous mass. This was removed by centrifugation after absorption, washed twice at 4° C. and treated with DNase in the usual manner. The second method was to add to SLE serum the same amount of DNA, but then conduct the absorption at 37° C. with considerable agitation so that the DNA dissolved in the serum. After absorption, an amount of protamine was added sufficient to precipitate most of the DNA. This precipitate formed instantaneously and was promptly spun out of the serum. The serum was then analyzed for L.E. cell activity and the DNA-protamine precipitate digested in the usual way. In neither instance was the DNA capable of absorbing any significant amount of L.E. cell factor, though in each case it absorbed γ-globulin which was liberated after the nuclease digestion alone.

Additional evidence supporting the participation of histone in the reaction with the L.E. cell factor emerges from the failure of the L.E. cell factor to displace histone upon combination with nucleoprotein. A solution of purified L.E. cell factor, recovered from nucleoprotein, was absorbed with a fresh aliquot of nucleoprotein which was then removed by centrifugation. After absorption, all but a trace of the γ-globulin and 90 per cent of the total protein had been removed,

TABLE V

*Absence of histone liberation from nucleoprotein during combination of L.E. cell factor and nucleoprotein*

| | γ-Globulin | Total protein | L.E. cell formation |
|---|---|---|---|
| | | *mg./0.5 ml.* | |
| L.E. cell factor solution | +++ | 0.310 | ++++ |
| L.E. cell factor solution after absorption | tr. | 0.032 | ± |
| Supernate from DNase digestion of absorption pellet | tr. | 0.050 (minus DNase) | ± |
| Supernate from 56° elution digestion residue | ++ | 0.110 | +++ |

TABLE VI

*Inhibition of reaction of L.E. cell factor with nucleoprotein**

| | | Untreated control nucleoprotein | Nucleoprotein pretreated with histone | Nucleoprotein pretreated with protamine | Nucleoprotein pretreated with atabrine |
|---|---|---|---|---|---|
| L.E. prep. with absorbed serum | | 0 | ++++ | ++++ | +++ |
| Supernate DNase digestion | γ-Globulin | ++ | 0 | 0 | + |
| | L.E. prep. | ± | | | |
| Supernate 56° elution | γ-Globulin | ++ | 0 | 0 | tr. |
| | L.E. prep. | ++++ | 0 | 0 | + |

* Nucleoprotein pellets were incubated for 60 minutes at 37° C. in solutions of histone, protamine or atabrine, washed and used to absorb SLE serum. See text for details.

leaving behind a small amount of L.E. cell factor (Table V). The fact that the total protein of that solution was reduced by at least 90 per cent suggests that the L.E. cell factor does not displace histone from nucleoprotein. After this second absorption, L.E. cell factor could again be recovered from the nucleoprotein in the usual way. The values for total protein given in Table V include both γ-globulin and undetermined amounts of histone. The failure to recover all of the L.E. cell factor absorbed by the nucleoprotein may be explained by the inability of the procedures to liberate all γ-globulin from the nucleoprotein complexes (Table II).

*Inhibition of the reaction between L.E. cell factor and nucleoprotein*

It had previously been found that exposure of cell nuclei to protamine or atabrine, which react with DNA and nucleoprotein to form stable compounds (22), prevented subsequent reaction of these nuclei with the L.E. cell factor (1). Similar experiments were conducted with nucleoprotein using histone, protamine and atabrine. Nucleoprotein pellets containing 1.2 mg. DNA were incubated for one hour at 37° C. in 1 ml. of isotonic saline solutions containing 1 mg. of histone or protamine or in a solution of isotonic saline saturated with atabrine. The pellets were then washed six times and used to absorb SLE serum. Little or no absorption of L.E. cell factor occurred (Table VI). The nature of the reaction of the inhibiting substance with nucleoprotein was not the same in each case. In histone solution, the nucleoprotein absorbed histone until there was an increase of approximately 70 per cent in the total histone of the nucleoprotein. No such absorption occurred in protamine solution; the total protein of the nucleoprotein diminished slightly. However, after incubation with nucleoprotein, the protamine solution contained a nondialyzable protein not present prior to the incubation. Because protamine is dialyzable and histone is not, it appears that protamine displaced some histone from the DNA, thus changing the original DNA-histone nucleoprotein at least partly into a DNA-protamine nucleoprotein. Atabrine stained the nucleoprotein yellow. For technical reasons it was not possible to establish clearly whether atabrine displaced histone. However, atabrine is known to react both with DNA and with albumin (22), and it is possible that it reacted with either DNA or histone of the nucleoprotein or both.

*Failure of L.E. cell factor of one patient to react with nucleoprotein saturated with the factor of another patient*

Successive aliquots of serum of one patient were absorbed with the same nucleoprotein pellet until no additional L.E. cell factor could be removed from the serum. The pellet was then washed and used to absorb another serum. In no instance was the pellet, saturated with the L.E. cell factor of one patient, able to absorb L.E. cell factor from the serum of another patient. These experiments were done with various combinations of the five sera used in the other experiments.

*Immunological characteristics and sedimentation behavior of the L.E. cell factor*

The data presented in this section were derived from the studies done with whole SLE serum and with L.E. cell factor recovered from nucleoprotein. Normal human γ-globulin consists of two main molecular species; approximately 90 to 95 per cent has a molecular weight of 160,000 and a sedimentation rate of approximately 7S, while the remaining 5 to 10 per cent has a molecular weight of about 1,000,000 and a sedimentation rate of approximately 19S (23). Figure 3 and Table VII indicate that the L.E. cell factor possesses the immunologic characteristics of typical human 7S γ-globulin. In Figure 3A, the isolated L.E. cell factor from one patient in Well 1 forms a single merging band of precipitate against antiserum to whole normal human serum and antiserum to normal human 7S γ-globulin. In Figure 3B the L.E. cell factor from another patient in Well 4 forms similar single bands of identity with normal 7S γ-globulin as both diffuse against the same antisera. The L.E. cell factors isolated from 20 different sera have all reacted with antiserum to normal human 7S γ-globulin. At no time has any serum protein other than γ-globulin been found in the L.E. cell factor solutions. Occasionally a small amount of the 19S γ-globulin has appeared, the significance of which is obscure.

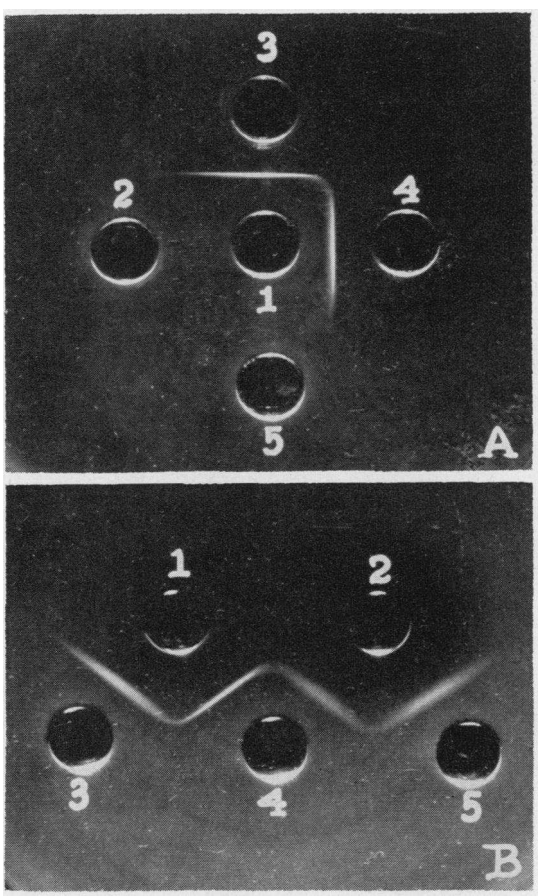


FIG. 3. REACTION OF PURIFIED L.E. CELL FACTOR WITH VARIOUS ANTISERA

3A. L.E. cell factor from Patient Ter. in Well 1; Well 2, antiserum to normal human 19S γ-globulin; Well 3, antiserum to normal whole human serum; Well 4, antiserum to normal human 7S γ-globulin; Well 5 antiserum to normal human albumin.

3B. L.E. cell factor from Patient Gar. in Well 4; Wells 3 and 5 contained normal human 7S γ-globulin; Well 1, antiserum to normal whole human serum; Well 2, antiserum to normal human 7S γ-globulin.

TABLE VII

*Inhibition of activity of L.E. cell factor by antiserum to normal human γ-globulin*

| L.E. cell formation by solution of purified L.E. cell factor | Incubated with | L.E. cell formation after incubation | γ-Globulin present after incubation (Cap. tube pptin.)* |
|---|---|---|---|
| ++ | Normal rabbit serum | ++ | ++ |
| ++ | Rabbit as normal human γ-globulin | 0 | 0 |

* Capillary tube precipitation.

Table VII shows that the capacity to induce L.E. cell formation is completely destroyed by antiserum to normal human γ-globulin. Equal aliquots of solution of purified L.E. cell factor were mixed with equal amounts of normal rabbit serum or rabbit antiserum to normal human γ-globulin. These mixtures were incubated one hour at room temperature and overnight at 4° C. The precipitates were then spun out and the supernates analyzed for the presence of γ-globulin and for L.E. cell inducing capacity. The supernates from the L.E. cell factor incubated with normal rabbit serum retained the L.E. cell inducing capacity and γ-globulin but the L.E. cell factor and the γ-globulin were precipitated completely from the mixture with antiserum.

The L.E. cell factor also possesses the sedimentation characteristics of human 7S γ-globulin.

This is seen in Table VIII. SLE serum was separated in a sucrose gradient by density gradient zone centrifugation (24). This procedure results in a separation of the high molecular weight γ-globulin from the low molecular weight γ-globulin, the former appearing at the bottom of the tube and the latter remaining near the top. After centrifugation, the contents of the tube were divided into five fractions from top to bottom. The material in each of these fractions was analyzed for protein content, for γ-globulin by a quantitative precipitin method, and for L.E. cell activity. It will be seen that the L.E. cell activity remained in that portion of the tube where the bulk of the γ-globulin with the lower molecular weight was found, and no L.E. cell activity was found in the lower portion of the tube where the high molecular weight γ-globulin accumulated. For comparison, in the final column is shown a similar separation of serum from a patient with rheumatoid arthritis where the rheumatoid factor, which is a high molecular weight γ-globulin, was found in the bottom of the tube.

### *pH and temperature stability of the L.E. cell factor and its complex with nucleoprotein*

Isolated L.E. cell factor was found to be stable in glycine buffers at pH 2 and pH 11 for at least 24 hours at 4° C. In isotonic NaCl, it was stable at 65° C. for at least 30 minutes; after exposure to 70° C. for 30 minutes some loss of activity occurred, while exposure to 75° C. for 30 minutes resulted in considerable though not complete loss of activity.

TABLE VIII

*Distribution of L.E. cell factor, γ-globulin, total protein and rheumatoid factor in fractions of serum separated by density gradient zone centrifugation**

| Fraction No. | Total protein | Immunological γ-globulin | L.E. cell formation | Sensitized sheep cell agglutination |
|---|---|---|---|---|
| | *mg./ml.* | *mg./ml.* | | |
| 1 (top) | 1.2 | 0.1 | | |
| 2 | 9.8 | 1.5 | +++ | 0 |
| 3 | 8.5 | 3.5 | ++++ | 0 |
| 4 | 2.6 | 0.9 | | 1/16 |
| 5 (bot.) | 0.6 | 0.2 | 0 | 1/512 |

* Two-tenths ml. serum layered onto 5 cc. of sucrose in a gradient varying from 10 per cent sucrose at the top to 37 per cent sucrose at the bottom. Ultracentrifugation for 18 hours at 110,000 G. resulted in sedimentation of 19S class proteins to the bottom of the tube. Contents of tube were then collected as five separate fractions by a stabilized capillary pipette. Titers given for sheep cell agglutination refer to dilution of fraction collected.

All experiments involving the complex of nucleoprotein and L.E. cell factor thus far reported were conducted near pH 7. An effort was therefore made to determine the stability of the complex at different pH's and at temperatures higher than 56° C. Complexes of L.E. cell factor and nucleoprotein were prepared and exposed to temperatures from 60° C. to 75° C. for 30 minutes in isotonic NaCl, or to pH 3.4, 5 and 8 in glycine buffers at 37° C. for 30 minutes. The complexes were then separated from supernates. The supernates were brought to pH 6.7 by dialysis against isotonic saline and tested for γ-globulin and ability to induce L.E. cell formation. The complexes were digested with DNase and eluted at 56° C. to recover the L.E. cell factor. Neither higher temperatures nor different pH's liberated L.E. cell factor from the complex. However, heating the complex above 60° C. or exposing it to pH 3 or below appeared to alter it in such a way as to prevent future recovery of the full amounts of γ-globulin or of active factor from the complex. Because nucleoprotein dissolves above pH 8, the influence of higher pH was not studied. It was also found that the L.E. cell factor and nucleoprotein can combine between pH 4 and pH 8. The effect of more extreme pH's upon their ability to combine was not explored.

### *Amount of L.E. cell factor in SLE serum*

SLE is characterized by hypergammaglobulinemia. However, the L.E. cell factor in the instances tested has comprised only a small fraction of the total γ-globulin. Figure 4 shows a determination of total serum γ-globulin by the quantitative precipitin method and by zone electrophoresis before and after removal of L.E. cell factor. Only slight diminution of γ-globulin occurred despite complete removal of L.E. cell factor from a highly positive serum by absorption with cell nuclei. In one such instance, the total γ-globulin was 20.5 mg. per ml. before and 19.8 mg. per ml. after absorption.

## DISCUSSION

The accumulated evidence strongly supports the view that the L.E. cell factor reacts with deoxy-

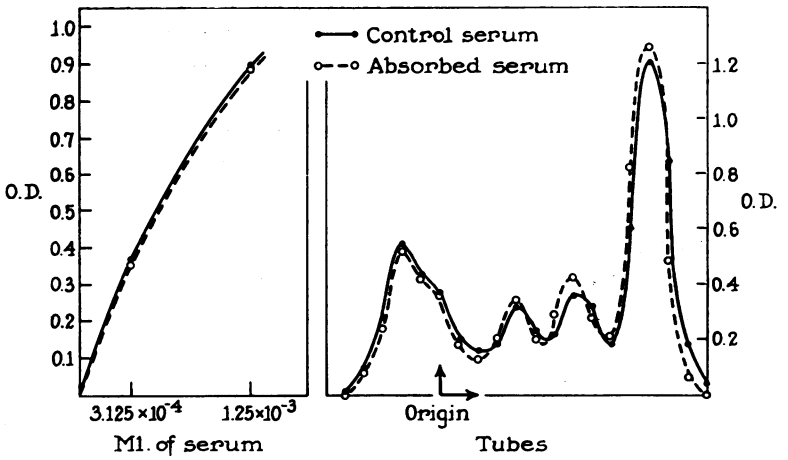


FIG. 4. EFFECT OF REMOVAL OF L.E. CELL FACTOR UPON TOTAL SERUM γ-GLOBULIN

Control serum refers to SLE serum before absorption. Absorbed serum has had L.E. cell factor entirely removed by absorption with cell nuclei. On the left is a quantitative precipitin curve using dilutions of the SLE serum and standardized rabbit antiserum to normal human γ-globulin. On the right is the serum protein pattern obtained by separation on starch in barbital buffer, ionic strength 0.1, pH 8.6. γ-Globulin is the peak to the left of the origin. Absorption caused very slight drop in γ-globulin.

ribonucleoprotein and requires both DNA and histone for reaction. This evidence includes: *1*) the two steps required for removal of the factor from the complex with nucleoprotein; *2*) the failure of the separated components of nucleoprotein or of purified DNA to react with the factor, and the partial appearance of activity on recombination of nucleoprotein components or on addition of histone to DNA; *3*) the inactivity of the complexes when long chain acids are substituted for DNA or when protamine is substituted for histone; and *4*) the apparent failure of the L.E. cell factor to displace histone from the nucleoprotein. The requirement of both DNA and histone is compatible with the results of current histochemical studies of the L.E. cell phenomenon (25).

The exact sites of combination of the L.E. cell factor and nucleoprotein remain obscure. In histochemical studies, exposure of cell nuclei to methyl green prior to absorption of the L.E. serum greatly diminished uptake of γ-globulin. Because methyl green is thought to react with the phosphate groups of DNA, it has been suggested that the L.E. cell factor also reacts with these groups. However, the inhibition of the reaction by prior exposure of nuclei to methyl green, or of nucleoprotein to histone or atabrine does not permit a conclusion on this question. It is possible that these substances interfere with the union by physical obstruction of access to the binding sites rather than by direct chemical reaction with these sites.

The biologic role of histone is unknown, and in native nucleoprotein histone may shape or hold the DNA in a particular configuration. Therefore, it is possible that in the reaction with the L.E. cell factor too, the role of the histone is to hold the DNA in proper configuration for binding and not to participate directly in the bond. However, the persistent attachment of the L.E. cell factor to the histone residue after DNase digestion of the complex argues for a direct combination between the factor and the histone.

The possible participation of the nonhistone protein of the nucleoprotein in the reaction is unsettled. Nonhistone protein comprises a minor but definite portion of the protein of nucleoprotein. It is not usually extracted by acid and it is probable that the protein which resisted extraction in these experiments was in part nonhistone protein. Since this residue absorbed a small amount of factor, reactivity of nonhistone protein cannot be excluded.

In the present experiments, the L.E. cell factors

from many different sera, including one from a patient with "apresoline disease," have reacted with nucleoprotein in an apparently identical way, all requiring both DNA and histone. This has been true regardless of reactivities which each individual serum showed toward other constituents of the nucleus. Furthermore, once nucleoprotein has been fully saturated with the L.E. cell factor from one serum, it has proved unable to absorb L.E. cell factor from another serum. Thus, while the possibility remains that L.E. cell factors may be found with different specificities, all L.E. cell factors thus far studied have been similar.

The requirement of DNA for reaction, and the apparent similarity of the L.E. cell factor in all sera studied, are at variance with the results of another study (14). The differences are probably a consequence of the methods used. In the latter study, the L.E. cell factor was not specifically identified. Instead, the absorption of γ-globulin from SLE sera onto nuclei was estimated by the fluorescent antibody method. All γ-globulin adherent to whole nuclei was assumed to be L.E. cell factor and the presence of other antinuclear antibodies was not considered. However, it is possible to explain all the results obtained on the basis of reactions of other antinuclear antibodies without inferring that the L.E. cell factor itself either differs from serum to serum, or that it does not require DNA. The existence of a group of antinuclear antibodies makes it necessary that techniques be employed which permit identification of the specific antibody in question if individual reactivities are to be studied.

The recovery of the L.E. cell factor from nucleoprotein permits a high degree of purification. The recovered solution of factor often exhibits approximately the same ability to cause formation of L.E. cells as an equivalent volume of original serum. In terms of γ-globulin concentrations, this has represented purification varying from 50- to 100-fold in different cases. No serum protein other than γ-globulin has ever appeared in the solutions of purified factor. The presence of other antinuclear factors has not been entirely excluded, though the solutions have not been able to fix complement with either pure DNA or the material extractable from nuclei with 0.1 ionic strength buffers and are presumably free of the factors reacting specifically with these materials.

The recovery of L.E. cell factor from nucleoprotein has permitted concentration of the factor and thus allowed search for the factor in sera which appear incapable of forming L.E. cells. This is conveniently done by absorbing a large amount of serum and eluting from the nucleoprotein with much smaller volumes. In preliminary experiments, L.E. cell factor has been recovered in this way from weakly positive or apparently negative sera. However, this procedure has not been done in enough cases, particularly in ones without clear clinical symptoms or complement fixation reactions of SLE, to gauge its ultimate usefulness.

Many previous experimental results appear to separate the L.E. cell factor from the factor which reacts with DNA alone. The latter factor is present far less frequently than the L.E. cell factor. It has been shown to precipitate and fix complement with pure DNA. Absorption of SLE serum with DNA alone has never appreciably reduced the capacity of the serum to form L.E. cells regardless of whether the amount of DNA used was equal to that found in equivalence point precipitates or was in excess of that amount. However, occasionally the γ-globulin recovered from equivalence point precipitates of DNA with SLE serum has shown very weak ability to form L.E. cells. Seligmann (13) has interpreted the latter finding to indicate a probable identity of the two factors.

In the present experiments, further evidence was obtained for the existence of two separate factors requiring DNA for reaction. Nucleoprotein absorbs both factors. The γ-globulin recovered from this nucleoprotein after DNase digestion alone fixes complement with pure DNA but has little if any ability to form L.E. cells. Conversely, the γ-globulin liberated by elution from the digestion residue forms L.E. cells readily but has not been capable of reaction with pure DNA. These results indicate that the DNA factor combines only with DNA of the nucleoprotein and is therefore liberated by DNase treatment alone. The L.E. cell factor combines with both DNA and histone and requires the two step procedure for liberation. Furthermore, repeated efforts to absorb the L.E. cell factor with purified DNA failed, though the factor which reacts with DNA alone was completely removed.

A likely explanation for the recovery of small amounts of L.E. cell factor from equivalence point precipitates obtained from mixtures of certain SLE sera and purified DNA would be a weak "cross reaction" between the L.E. cell factor and DNA alone. This would be consistent with the behavior of an antibody toward a substance which contains some but not all of the required combining sites. It is also possible that L.E. cell factors possess different relative affinities for DNA and histone, or that the factor which reacts with purified DNA can occasionally cause nuclear changes similar to those caused by the L.E. cell factor.

The serum factor which precipitates or fixes complement with pure DNA is present in a minority of SLE sera. Many of the SLE sera used in this study did not show these reactions with pure DNA. However, when they were absorbed with nucleoprotein and the nucleoprotein was digested with DNase, γ-globulin was liberated. Despite recovery in significant amounts, this γ-globulin was incapable of fixing complement or precipitating with DNA, or of causing L.E. cell formation. In view of the fact that such a γ-globulin has not been recovered after absorption of normal serum, it appears probable that γ-globulins with an affinity for DNA but which are incapable of precipitation or complement fixation with DNA are commonly present in SLE serum. Support for this interpretation comes from the fact that absorption of such SLE sera with pure DNA followed by precipitation of the DNA with protamine and then digestion of the DNA-protamine complex with DNase, also yields this type of γ-globulin. The reaction of this latter γ-globulin with DNA appears to be specific because it occurs above pH 7.2, which is above the isoelectric point of both γ-globulin and DNA. Therefore the net charge on both molecules would be negative, and electrostatic interactions would not be a likely cause of the reaction.

The evidence thus far accumulated suggests that the reaction of the L.E. cell factor and nucleoprotein is an immune reaction. The isolated factor has the physical and immunological properties of typical human antibody γ-globulin. It appears to have a sharply defined requirement for reaction. It is one of a group of antinuclear factors all of which are identified by standard immunological techniques. The absence of species or organ specificity exhibited by these antinuclear factors is not characteristic of many immune systems, but antibodies to pneumococcal polysaccharides possess a very broad carbohydrate reactivity (26), and antibodies to lens, brain and testes do not show species specificities (27).

The main deficiency in the evidence for an immune reaction lies in the great difficulty in obtaining analogous factors after immunization of animals with nuclear constituents (28). However, Miescher has attained some success (29). The difficulties may be due to the absence in the animals of the abnormal immune system which appears to be present in patients with SLE.

The pathogenic significance of the antinuclear factors is unknown. Considerable evidence suggests that these factors cannot enter a normal cell *in vivo* (4). They therefore appear to be by-products of an abnormal immune system rather than responsible pathogenic agents. However, exploration of this question is in its infancy.

## SUMMARY

1. The lupus erythematosus (L.E.) cell factor reacts with deoxyribonucleoprotein of the cell nucleus and requires both deoxyribonucleic acid and histone for the reaction. This reaction appears to be the primary step in formation of the L.E. cell.

2. The L.E. cell factor can be isolated from its complex with nucleoprotein by digestion of the complex with deoxyribonuclease followed by elution from the residue.

3. The L.E. cell factor possesses the immunological and physical properties of typical human antibody γ-globulin.

4. The reaction of nucleoprotein with L.E. cell factor can be inhibited by prior exposure of the nucleoprotein to histone, protamine and atabrine. The mechanism of those inhibitions is not established.

5. The properties of the L.E. cell factor and the specific requirement of deoxyribonucleoprotein for reaction suggest that the reaction is immunological, and that L.E. cell factor is one of a group of antibodies to constituents of the cell nucleus which arise in systemic lupus erythematosus.

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