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FLOURESCENT ANTIBODY IN THE STUDY OF LUPUS ERYTHEMATOSUS SERUM

MELVILLE P. ROBERTS JR

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THE USE OF FLUORESCENT ANTIBODY AND ISOLATED CHROMOSOMES IN THE STUDY OF LUPUS ERYTHEMATOSUS SERUM

Melville P. Roberts, Jr.

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INTRODUCTION

From the time when the lupus erythematosus cell was first described by Hargraves, Richmond, and Morton[1] there has been much speculation as to what factors are responsible for its production. Initially it was believed that the L.E. cell was was pathognomonic of lupus erythematosus.[2] As the phenomenon became better known, however, various workers reported the L.E. cell phenomenon in other diseases.[3,4,5,6]

Early investigators of the L.E. cell hypothesized that the phenomenon was due to a factor present in the blood plasma. This theory was later borne out by experiments which demonstrated that the L.E. factor was present in the gamma globulin fraction of the plasma proteins.[7]

Miescher has demonstrated that absorption of lupus serum with isolated nuclei results in removal or destruction of the L.E. factor.[8] Very recently the fluorescent antibody technique has been applied to this problem. In the first report of such studies it was shown in vitro that a globulin factor of lupus serum adheres to nuclei in sections of tissue.[9] Further studies revealed that nucleoprotein might be the nuclear factor involved in this reaction.[10] The investigation to be reported here is an attempt

to further elucidate this problem through the use of isolated chromosomes and the fluorescent antibody technique.

The L.E. Cell

The L.E. cell was first described by Hargraves, Richmond, and Morton in 1948. They often noted that in the bone marrow of patients with lupus erythematosus a peculiar cell was to be found. "This cell undoubtedly represents a process which is going on in such bone marrows and is the end result of one of two things: either phagocytoses of free nuclear material with a resulting round vacuole containing this partially digested and lysed nuclear material or, second, an actual autolysis of one or more lobes of the nucleus of the involved cell so that it presents essentially the same appearance as the one which has phagocytized nuclear material."[1] Hargraves, Richmond, and Morton concluded their description of the L.E. cell by suggesting that the finding of L.E. cells in a patients' bone marrow might be of some help in diagnosing lupus erythematosis. Shortly after the publication of the original description other workers reported finding such cells in patients with systemic lupus erythematosis.[11]

For a time it was believed that these peculiar cells were pathognomonic of disseminated lupus erythematosis.[2,16] Later investigations, however, demonstrated that this was not true. L.E. cells have been found in association with the "collagen diseases" and with drug hypersensitivity reactions

as well. In particular, L.E. cells have been described in periarteritis nodosa,[3] penicillin[4] and hydantoin[5] hypersensitivity, as well as during hydralazine therapy.[6]

The L.E. Cell Phenomenon

Until 1949 the only reliable way in which L.E. cells could be demonstrated was by aspiration and examination of bone marrow. If the bone marrow was hypoplastic or the patient too ill for sternal puncture this method of investigation had obvious disadvantages. In 1949 Haserick and Bortz found that L.E. cells could be produced by adding plasma taken from patients with acute disseminated lupus erythematosis to heparinized normal bone marrow.[12,13] It was also noted that leukocyte clumping was induced.

The discovery of the L.E. cell phenomenon was very important because it shed light on a few basic principles concerning the L.E. cell. First of all, it revealed that the plasma of patients with acute disseminated lupus erythematosus contained a factor responsible for the formation of the L.E. cell. Secondly, it also became apparent that the production of the L.E. cell was a secondary rather than a primary phenomenon.

The L.E. Cell Factor

Now that it had been established that the formation of L.E. cells was due to the presence of a factor in the plasma the next step was to identify the substance. A few facts were already known concerning its nature. Firstly, it was stable for periods of at least as long as six months provided that the plasma was kept sterile.[11] It was destroyed by heating the plasma to 65° C. Para-aminobenzoic acid seemed to inhibit the activity of the factor, although this may have been due to a direct effect of the acid on the bone marrow. The factor could not be detected in patients during a remission but only during the acute phase of the disseminated lupus erythematosus. The factor could not be found in patients suffering from rheumatic fever, subacute or chronic lupus erythematosis, cirrhosis, rheumatoid arthritis, scleroderma, dermatomyositis, or periarteritis nodosa.[14] Later workers, however, were able to demonstrate the L.E. phenomenon in some of these conditions and in other diseases as well.

In order to identify which portion of the blood plasma contained the L.E. factor, Haserick, Lewis, and Bortz in 1950, employed fractionation by the Tiselius electrophoretic technique.[7] These investigators divided plasma from a patient with acute

disseminated lupus erythematosis into several fractions: 1) albumin, (3.5 mg.); 2) albumin, alpha and beta globulin (12 mg.); 3) gamma globulin (2 mg.); and 4) gamma globulin and fibrinogen (4.8 mg.). Then, by adding each fraction to normal bone marrow and noting L.E. cell formation, it was determined that only the gamma globulin fraction contained the L.E. factor.

In an attempt to prove that the L.E. factor is an immunologically distinct component of gamma globulin, Haserick and Lewis conducted an ingenious experiment.[15] By electrophoresis they first separated the gamma globulin fraction from the plasma obtained from a patient suffering with acute disseminated lupus erythematosis. An antiserum against this fraction was then produced by repeated injections of the gamma globulin into rabbits. A control rabbit antiserum against normal human gamma globulin was also produced by the same method. Next, the investigators took a quantity of human L.E. gamma globulin and divided it into two portions. To one portion was added the antiserum to L.E. gamma globulin, and to the other was added the control antiserum. In both portions a precipitate formed and was removed by centrifugation. The supernatant

fluid from each portion was then added to bone marrow preparations which were subsequently examined for L.E. cell formation. The L.E. gamma globulin to which had been added the antiserum to normal human gamma globulin caused the production of L.E. cells. On the other hand, the L.E. gamma globulin to which had been added antiserum to L.E. gamma globulin was unable to induce L.E. cell formation.

Although the above experiment has been widely accepted as proof that the L.E. factor in gamma globulin is a distinct immunological entity, the possibility of incomplete precipitation remains as an alternative explanation.

At this point it would be appropriate to list the salient properties of the L.E. factor as revealed by past investigations.

1. The L.E. factor is contained in the gamma globulin fraction of the blood plasma.
2. The presence of the factor can be demonstrated by adding L.E. plasma to a normal bone marrow preparation thus producing L.E. cell formation (this is referred to as the L.E. cell phenomenon).

3. The L.E. factor is probably a distinct immunological entity.
4. The L.E. factor has been found in diseases other than acute disseminated lupus erythematosis, notably in drug hypersensitivities and in some of the other "collagen diseases."
5. The factor induces nucleolysis and nucleophagocytosis with the resulting production of an L.E. cell.

Methods and Materials

The Fluorescent Dye Labeled Antibody Technique

The technique of labeling an antibody with a dye so that the complex resulting from the combination of this labeled antibody with its specific antigen could be visualized was first successfully accomplished by Reiner in 1930.[17] Reiner was able to chemically combine antipneumococcus I and II antibodies and atoxyl-azo dyes. He noted that the dye-antiserum complex had the same agglutinative titre as the original antiserum and that its mouse-protection activity was unaltered.

Marrack, in 1934, reacted diazotized benzidine-azo-R-salt with anti-typhoid and anti-cholera sera and then demonstrated that the homologous organisms were stained pink by the dye-labeled antibodies.[18] In order to prove that the dye-typhoid antibody complex was immunologically identical with the original typhoid antiserum he performed a rather clever experiment. He mixed ordinary cholera antiserum with an equal amount of the typhoid antiserum-dye complex. To one-half of this mixture he then added typhoid bacilli. The cholera vibrios were agglutinated but unstained. The typhoid bacilli, on the other hand, were not only agglutinated but also stained red. This simple procedure demonstrated

that the typhoid antiserum was immunologically unaltered by the dye conjugation.

Coons, Creech, and Jones were the first to combine an antiserum with a fluorescent moiety and thus render the resulting antigen-antibody complex fluorescent in ultraviolet light.[19] In 1941 they prepared a B-anthryl-carbamido derivative of anti-pneumococcus III rabbit serum which produced a faint blue fluorescence in daylight and an intense blue fluorescence in ultraviolet light. At the same time it was demonstrated that the conjugation procedure in no way interfered with the specific reactivity of the antiserum.

In 1942, Coons and his group showed that a fluorescein-carbamido-pneumococcal III antiserum could specifically stain tissues from a mouse infected with type III pneumococcus.[20] In 1950, Coons and Kaplan reported improvements in the synthesis of fluorescein isocyanate which had been first accomplished by Bogert and Wright[21,22] and modified later by Coons, Breech, Jones, and Berliner.[23] At the same time they also defined the optimum conditions for combining the fluorescent isocyanate dye with protein materials. They then conjugated antipneumococcal rabbit serum, type III, with fluorescein

isocyanate II by their improved technique. The fluorescent dye-labeled antipneumococcal antiserum was then successfully used to locate pneumococcal polysaccharides in the tissues of mice.[24]

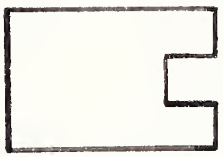
Many other investigators have employed the Coons technique of combining antiserum with the fluorescent dye, fluorescein isocyanate, in order to locate a specific antigen in animal tissues. Conjugates have been prepared to identify the capsular polysaccharide of Friedlander's bacillus,[25] mumps virus,[26] plasma proteins,[27] Endamoeba histolytica,[28] and many other antigenic substances.

Figure I is a diagrammatic representation of the Coons dye labeled antibody reactions.

A slightly more complex, but just as useful technique is often employed. If, for example, one is dealing with an antigen, the antibody to which is located in the gamma globulin fraction of human serum it is not necessary to make up a specific dye-antibody conjugate against the antigen. Instead, a fluorescent antibody is made up against human gamma globulin. Then, any antibody from the gamma globulin which combines with the antigen will, in turn, also combine with the fluorescent antibody against the

Figure I

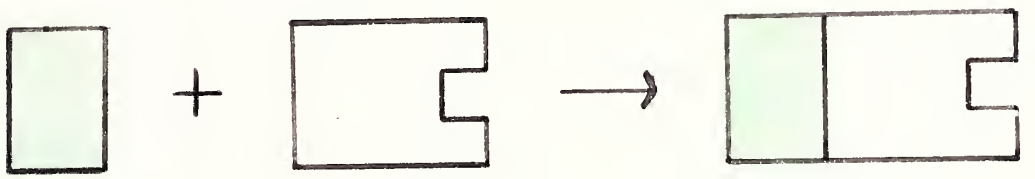
a. The antibody with its specific receptor, 1,



1.

Antibody

is chemically combined with a fluorescent dye, fluorescein isocyanate.



Dye (fluorescent)

Antibody

Dye-antibody conjugate (fluorescent)

b. To identify the specific antigen the dye-antibody conjugate is then added:

Dye-antibody conjugate (fluorescent)

Antigen

Conjugate-antigen complex (fluorescent)

human gamma globulin. Thus the original antigen is labeled by a two stage process (figure II).

In the work to be reported here, the techniques just described were employed in attempts to demonstrate that a factor in lupus serum is capable of combining with some material present in isolated chromosomes.

The Isolation of Chromosomes

The chromosomes were isolated from chicken erythrocytes, which are nucleated. The method used was that of Mirsky and Ris.[29] Blood was obtained by cutting the chicken's throat and collecting it in a bottle with oxalate. The blood (20 ml.) was then centrifuged for 15 minutes at 5000 rpm. at a temperature of one degree centigrade. The buffy coat and plasma were decanted and the remaining red cells washed three times in 0.14 M NaCl. Next, the washed cells were mixed with approximately 100 ml. of 0.14 M NaCl, placed in a Waring blendor, and beaten for 20 minutes. Ice was packed around the outside of the blendor so that the contents could be kept cool. The resulting mixture was centrifuged for 15 minutes at

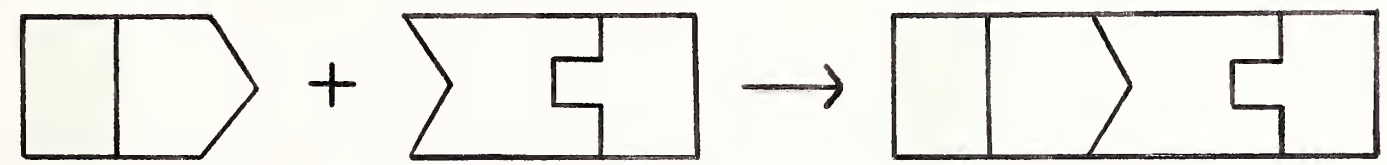
FIGURE II

a. Antibody with its specific receptor 1, and its species specific receptor, 2.

b. The antibody against the gamma globulin species specific recptor of antibody A is combined with a dye, fluorescein isocyanate.

c. To identify the specific antigen, antibody A (non-fluorescent) is first added.

d. Next, the dye-antibody B conjugate which is an antibody against the species specific receptor of antibody A is added.



5000 rpm, the red supernatent fluid poured off, and the precipitate, consisting of chromosomes which have been removed from the erythrocyte nucleus, then washed with saline. All solutions were kept at approximately one degree centigrade during the entire process.

To verify the presence of chromosomes in the precipitate, smears were made on slides and stained by the Fuelgen method which is specific for nucleoprotein. Chromosomes with their characteristic morphology, as described by Mirsky and Ris, could be identified.[26]

Source of Human Sera

Serum was obtained from patients who had been diagnosed clinically as suffering from lupus erythematosus. The sera used for these tests was known to contain the factor, described by Friou, which reacts with intact nuclei. Normal serum was also obtained. The serum was frozen and stored at minus ten degrees centigrade and thawed immediately before use.

The Fluorescent Antibody*

Anti-human globulin was produced by repeated injection of an alum precipitated gamma globulin

* The fluorescent conjugated antiglobulin was provided by Dr. George J. Friou.

into rabbits. The anti-human globulin was then separated by ammonium sulfate fractionation from the rest of the rabbit serum. Next, the anti-human globulin was conjugated with fluorescein isocyanate II. The procedure used was that of Coons and Kaplan as described in their paper of 1950.[21] The resulting fluorescent rabbit anti-human globulin proved to have a precipitin titre of 1:10 against human globulin.[30]

The Staining Procedure

A drop of the chromosome preparation was placed on each of a series of slides. The slides were dried at room temperature for ten minutes at a distance of three feet from an electric fan. After the drying process had been completed, one drop of the thawed lupus erythematosus serum was applied to the dried chromosome precipitate on each slide. Next, the slides were incubated for one half hour at room temperature. To prevent drying during the incubation period these were placed in a covered pan with a moist towel in the bottom. The following step was a washing procedure using phosphate buffer prepared by adding 40 grams of sodium chloride to 6.9 grams of sodium phosphate and mixing with enough distilled water to give a volume of 5000 cc. The pH was

adjusted to 7.0 with 40 per cent NaOH.

After the washing has been completed, the slides were shaken once or twice to remove any excess buffer. One drop of the fluorescent rabbit anti-human globulin conjugate was then applied and the slides incubated for one-half hour again, in the covered pan. The washing procedure was repeated. Then, one drop of a glycerine-phosphate buffer solution (1 ml. of phosphate buffer plus 9 ml. of glycerol) was added to each slide and cover slips applied. The slides were now ready to be examined.

Results

The chromosomes were seen to readily fluoresce under the ultraviolet microscope (plate 1.). This suggests that a specific complex has been formed in the following sequence: first the chromosomes bind a factor in the gamma globulin fraction of the lupus erythematosus serum, then the fluorescent rabbit anti-human gamma globulin attaches to the gamma globulin. The latter combination is known to be an antigen-antibody reaction.

Two controls were run in order to prove that a specific combination had taken place between some factor in the L.E. serum and the chromosomes.

PLATE I

Chromosome stained with fluorescent antibody. Due to the printing process the stain appeares blue rather than the original green.

1.) Normal serum was substituted for the L.E. serum in the staining process. No fluorescence was observed. 2.) L.E. serum was absorbed with nucleoprotein in the following manner: calf thymus nucleoprotein was precipitated by adding 170 ml. of .177 M sodium chloride solution to 100 ml. of calf thymus nuclear extract and refrigerating at 40 degrees F. for one-half hour. The precipitate was then washed with .15 M sodium chloride solution and centrifuged. The absorption was accomplished by adding the nucleoprotein precipitate to 5 ml. of L.E. serum and letting the mixture stand at 40 degrees F. One hour later the L.E. serum was decanted and the process repeated with another batch of nucleoprotein precipitate. It was then determined that the absorbed serum did not produce the L.E. phenomenon nor contain the factor which reacts with intact nuclei.[30]

When the absorbed lupus serum was used in the fluorescent staining process with chromosomes no staining took place. This suggests that the material in chromosomes with which the lupus globulin factor reacts is nucleoprotein.

The final step in the investigation confirms the impression that the material in chromosomes

which combines with the lupus serum factor is indeed nucleoprotein. Mirsky and Ris found that chromosomes could be fractionated into two parts by placing them in a neutral solution of 1 M sodium chloride.[29] One fraction, the supernatent fluid, proved to be nucleoprotein. The remaining insoluble precipitate, making up 8 to 10 per cent of the chromosomal mass contained, "12 to 14 per cent ribose nucleic and about one-fifth as much desoxyribose nucleic acid."[29] Following the method of Mirsky and Ris, the chicken erythrocyte chromosomes were fractionated. The supernatant fluid, virtually pure nucleoprotein, was dried on a slide and stained by the fluorescent antibody technique as outlined earlier. The nucleoprotein stained brightly.

Summary

By use of Coons' fluorescent antibody staining technique, it was demonstrated that a serum factor from patients with lupus erythematosus adheres to isolated chromosomes. It was further shown that this is due to a combination between the nucleoprotein of the chromosomes and the factor of the serum.

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