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DISSEMINATED LUPUS ERYTHEMATOSUS, SCLERODERMA, AND DERMATOMYOSITIS AS MANIFESTATIONS OF SENSITIZATION TO DNA-PROTEIN

I. AN IMMUNOHISTOCHEMICAL APPROACH *

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The semantic concepts of "connective tissue disease" and "collagen disease," proposed respectively by Klinge[1] and by Klemperer, Pollack and Baehr,[2] have stimulated many investigations of the normal and pathologic physiology of the connective tissues and have contributed significantly to the body of knowledge in this confusing area. In the past, however, attention has been focused primarily upon the late stages of the "collagen diseases" and their structural manifestations, notably fibrinoid degeneration or necrosis, and cicatrization. Disseminated lupus erythematosus, scleroderma, and dermatomyositis, little understood members of the group, have been conspicuous subjects for retrospective analysis.

The present investigation rests upon an examination and attempted elucidation of certain apparent antecedents to the fully developed lesions seen in these diseases. Although the evidence bearing upon the nature of collagen disease has been largely hypothetical, a basis in hypersensitivity has been implied sufficiently often to warrant further attention. Indeed, the recent work of Haserick, Lewis and Bortz,[3] incriminating gamma globulin in the development of the LE cell phenomenon, has provided a substantial spur in the same direction. The major premise of the present inquiry has been derived from the well known association between circulating antibodies and the gamma globulin fraction of serum protein. The possibility that collagen diseases, and particularly those mentioned above, might follow a hyperimmune reaction, has prompted an exploration of serums from patients

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afflicted with the specified disorders for the presence of specific antibody.

Gamma globulin and raw serums from individuals with various so-called collagen diseases have been employed as histochemical stains against autologous, homologous, and heterologous tissues, by the fluorescent antibody techniques developed by Coons and associates.[4-7] A binding by tissue components has been taken as presumptive evidence of local antigen-antibody precipitation, reflecting the presence of either true circulating antibody or an entity possessing similar traits within the original test serum.

Preliminary experimental results have revealed a bizarre but nonetheless reproducible reaction between cellular nuclei in human and animal tissues and an active agent seemingly present in the serum gamma globulin fractions of patients with disseminated lupus erythematosus (DLE), scleroderma (SCL), and dermatomyositis (DMS), as well as in cases of clinical rheumatoid arthritis presenting positive LE cell tests. Positive reactions have been characterized by a staining of nuclear material by test serum globulin labeled with fluorescent dye, when viewed in a fluorescence microscope under ultraviolet light. The specific nature of this hitherto unreported histochemical localization is not wholly clear, but some of the more provocative interpretations are discussed below and related to problems of pathogenesis and clinical application. Recently, we have become aware of similar investigations in progress elsewhere; only abstracts are at hand, however, and a detailed comparison of data must, therefore, be postponed.[8,9]

Materials and Methods

Clinical Material

Serums. Forty-three samples of serum were collected from 38 selected patients at the following institutions: Robert Breck Brigham Hospital (R.B.B.H); Boston Lying-in Hospital (B.L.I.H.); House of the Good Samaritan (H.G.S.); Children's Medical Center; Massachusetts General Hospital (M.G.H.); and Veterans Administration Hospital at West Roxbury, Massachusetts (V.A.H.). Within the collagen disease group, specimens were obtained from patients with acute disseminated lupus erythematosus (5 cases), discoid lupus (1 case), scleroderma (6 cases), dermatomyositis (2 cases), rheumatoid arthritis (8 cases), temporal arteritis (2 cases), and glomerulonephritis (1 case). A single serum specimen from a patient suffering from a penicillin reaction was also examined. Controls included serum from young and old individuals of both sexes, normal women in early and late stages of pregnancy, and patients with pre-eclamptic toxemia, acute

TABLE I

Synopsis of Staining Reactions

Patient*	Diagnosis	Source	LE prep.	Tissues: Human: Skin Dir.	Skin Indir.	Placenta Dir.	Placenta Indir.	Breast	Rabbit: Kidney	Myocardium	Lung	Adrenal
SCL Ia	Scleroderma, eclampsia	B.L.I.H.	−	+	+	+	+	o	o	o	o	o
SCL Ib	Scleroderma, eclampsia	B.L.I.H.	−	+	+	+	+	−	+	−	−	o+
SCL Ic	Scleroderma, eclampsia	B.L.I.H.	−	+	+	+	+	+	+	−	−	−
SCL Id	Scleroderma, eclampsia	R.B.B.H.	−	o	+	o	+	o	−	±	−	−
SCL IIa	Scleroderma	R.B.B.H.	−	o	+	o	+	+	+	+	+	+
SCL IIb	Scleroderma	R.B.B.H.	−	+	+	+	+	o	+	+	+	+
SCL III	Acrosclerosis	R.B.B.H.	−	o	+	o	+	−	+	+	+	+
SCL IV	Scleroderma	Private	−	o	+	o	+	o	±	±	+	+
SCL V	Atypical scleroderma	R.B.B.H.	−	o	−	o	−	o	−	−	−	−
SCL VI	Inactive scleroderma	R.B.B.H.	−	o	±	o	−	−	−	−	−	−
DLE I	D. L. E.	R.B.B.H.	+	+	+	+	+	o	+	+	+	+
DLE II	D. L. E.	R.B.B.H.	+	o	+	o	+	+	−	+	−	+
DLE III	D. L. E.	R.B.B.H.	+	o	+	o	+	o	+	+	+	+
DLE IV	D. L. E.	H.G.S.	+	o	+	o	+	o	+	+	+	+
DLE V	D. L. E.	H.G.S.	+	o	+	o	+	o	+	+	+	+
DLE VI	Discoid L. E.	B.L.I.H.	−	o	−	o	−	−	−	−	−	−
DMS I	Dermatomyositis	Private	o	o	+	o	+	o	+	+	+	+
DMS II	Dermatomyositis, inactive	R.B.B.H.	o	o	−	o	−	−	−	−	−	±
RhA Ia	Rheumatoid arthritis	R.B.B.H.	−	o	−	o	−	−	−	−	−	−
RhA Ib	Rheumatoid arthritis	R.B.B.H.	−	o	−	o	−	−	−	−	−	−
RhA II	Rheumatoid arthritis	R.B.B.H.	−	o	−	o	−	−	−	−	−	−
RhA III	Rheumatoid arthritis	R.B.B.H.	+	o	+	o	−	−	−	±	−	−
RhA IV	Rheumatoid arthritis	R.B.B.H.	−	o	+	o	−	−	−	−	−	±
RhA V	Rheumatoid arthritis	R.B.B.H.	+	o	+	o	+	o	+	+	+	+
RhA VI	Rheumatoid arthritis	R.B.B.H.	−	o	+	o	+	o	+	+	+	+
RhA VII	Rheumatoid arthritis	R.B.B.H.	+	o	−	o	−	o	−	−	−	−
RhA VIII	Rheumatoid arthritis	R.B.B.H.	+	o	+	o	+	o	+	+	+	+
TA I	Temporal arteritis	M.G.H.	o	o	−	o	o	o	−	−	−	−
TA II	Temporal arteritis	M.G.H.	o	o	−	o	o	o	−	+	−	−
GN I	Glomerulonephritis	V.A.H.	o	o	−	o	−	−	−	−	−	−
PR I	Penicillin reaction	V.A.H.	o	o	−	o	−	−	−	−	−	−
Controls	12 cases including instances of hydatidiform mole, toxemia, eclampsia, normal pregnancies, other male and female patients of various ages							Includes 4 conjugates for direct staining Results uniformly negative				

Key: + Nuclei stained ± Weak or irregular stain
— Nuclei not stained o Not performed

*Patients coded by literal abbreviations for clinical diagnosis, followed by Roman numerals assigned in chronological order. Lower case letters represent successive serum samples from same patient.

eclampsia, and hydatidiform mole. A detailed listing appears in Table I. Each case was classified according to prevailing clinical and pathologic diagnostic standards, and most patients were seen personally by one or another of the authors.

Blood drawn from each patient under sterile conditions was reduced to serum, distributed in small aliquots, quick-frozen in an acetone-dry ice mixture, and stored at −17° C. until required.

Tissues. Fresh surgical specimens, representing a variety of normal human tissues, were secured at the Robert Breck Brigham, Peter Bent Brigham, and Boston Lying-in Hospitals, and at the Children's Medical Center. Fresh samples of rabbit tissue, including the major organ systems, were used in addition. Necropsy material, although employed in the initial phases of the study, was found to be unsatisfactory. Each portion of tissue, as excised, was placed in a test tube and quick-frozen. Samples were stored in a deep freeze unit, and hardened over night in dry ice before cutting.

Preparation of Serum Conjugates

Globulin Fractionation. Serums were separated into crude globulin fractions at 0° C., with gentle mechanical stirring, according to the procedure of Coons.[10] An initial sample, generally about 20 to 40 ml., was diluted with an equal volume of normal saline. Saturated ammonium sulfate was added drop by drop with stirring, yielding a solution half saturated with respect to the precipitant. The turbid protein suspension was stirred for some time, and then stored over night in the cold to permit the reaction to reach completion. After centrifugation and washing with half-saturated ammonium sulfate, the globulin was dissolved in saline to approximately one third of the original volume, and then dialyzed in the cold against saline buffer[4] to remove both sulfate and ammonium nitrogen. The opalescent protein solution was protected with merthiolate in a final concentration of 1:10,000 and stored at 4° C.

Conjugates. The globulin fractions were conjugated to fluorescein isocyanate as described elsewhere.[4] Absorption, as performed in the classical procedure, was omitted to prevent removal of active principle by tissue nuclei. Globulin fractions and conjugates were spot-checked for purity by paper-strip electrophoresis; patterns indicated concentration in the gamma globulin region, with only traces of albumin. The latter did not interfere perceptibly with later procedures. Conjugates against human albumin (AHA; supplied by Dr. D. Gitlin, Children's Medical Center, Boston, Massachusetts) and gamma globulin (AHG; supplied by Dr. C. Liu, Department of Bacteriology and Immunology,

Harvard Medical School, Boston, Massachusetts) were prepared by active immunization of rabbits, while that formed against rabbit gamma globulin (ARG; supplied by Dr. B. K. Watson, Department of Bacteriology and Immunology, Harvard Medical School) utilized goat antiserum. The fluorescent conjugates used in direct staining procedures are catalogued in Table II.

TABLE II

Fluorescent Conjugates for Direct Staining

Code	Patient	Diagnosis
SCL Ia-F	SCL I	Scleroderma; eclampsia 2 mos. post partum
SCL Ib-F	SCL I	Scleroderma; eclampsia, acute
SCL Ic-F	SCL I	Scleroderma; eclampsia 5 mos. post partum
SCL IIb-F	SCL II	Scleroderma
DLE I-F	DLE I	Disseminated lupus erythematosus
C I-F	C I	Control; early normal pregnancy
C II-F	C II	Control; term normal pregnancy
C III-F	C III	Control; pre-eclamptic toxemia
AHG		Rabbit anti-human gamma globulin
AHA		Rabbit anti-human albumin

Cases other than those listed here have been investigated by indirect technique. For any serum specimen, the corresponding conjugate prepared for direct staining reactions has been designated by an upper case "F" following the serum sample number: SCL IIb-F.

Tissue Preparation

Sectioning. Frozen and prehardened blocks were cut at 5 μ in a cryostat at −17° C. After thawing on a fingertip and air drying at room temperature under a fan, the unstained sections were stored in the cold.

Fixation. Most stains were performed upon unfixed material. Other tissues were fixed in 5 per cent formalin or 95 per cent ethanol for 5 minutes at 37° C. Absolute methanol and anhydrous acetone, 10 minutes at 37° C., were also used.

Histochemical Procedures

Staining. Although unstained material was set aside occasionally for as long as 3 days after cutting to determine stability, sections generally were stained within an hour after preparation. Both the direct and the indirect or layering methods were used.

In the direct technique, tissues were stained with specific conjugates containing fluorescein-labeled gamma globulin, and local precipitation of fluorescent material was presumed to indicate sites of immune reaction. When fixed, tissues were dried in air at 37° C. for 30 minutes. After a 5 minute wash in saline buffer at room temperature, each slide

was laid flat and a drop of the proper conjugate spread over the tissue. The section was incubated with tagged protein for 20 minutes, whereupon the stained slides were washed once again in buffer for 10 minutes, to elute uncombined conjugate. A clean cover slip was mounted on each slide with a drop of buffered glycerol.[4]

The indirect method consisted of bathing the tissue with unconjugated serum, washing, and then staining with labeled anti-gamma globulin antiserum to localize any specific antibody precipitated from the serum during the first step. Because of a probable multiplication of binding sites when fixed antibody serves in turn as antigen, this technique is considered somewhat more sensitive.[7] One drop of the serum to be evaluated for antibody content was incubated with the tissue under a humidifier for 20 minutes, whereupon the section was washed 5 minutes in saline buffer to remove uncombined protein, and then stained for 30 minutes with a conjugate against gamma globulin. The section was washed once again in saline buffer for 5 minutes, mounted in buffered glycerol, and examined. When human serums were tested, the sections were counterstained with conjugated rabbit anti-human gamma globulin antiserum globulin (AHG), with conjugates AHA and ARG as controls.

Antigen Removal Tests. Specific enzymatic digestions were performed upon tissue sections; in most instances, a drop of enzyme solution was incubated on the tissue for 1 to 5 minutes at room temperature, followed by washing with saline buffer. Crystalline deoxyribonuclease (Nutritional Biochemicals Corp., Cleveland, Ohio) was used at concentrations of 0.01 to 1.0 mg. per ml. of buffer at pH 7.1. Crystalline bovine ribonuclease (Worthington Biochemical Corp., Freehold, New Jersey) was employed at a maximum concentration of 1.0 mg. per ml. Both enzymes were made up in the special phosphate-carbonate buffer* recommended by Wilbur and Anderson.[11,12]

Light trypsinization of the tissues was carried out to yield either destruction of the reactant or enhancement of the reaction; the latter possibility was suggested by the work of Coffin and Pickles,[13] who demonstrated the ability of trypsin to restore the specific agglutinating capacity of periodate-treated erythrocytes. Trypsin, as a solution of Viokase P-130D diluted to 1:100, was incubated with the tissue at 37° C. for varying periods up to 15 minutes. The results of the several digestions are shown in Table III.

* Composition of special phosphate-carbonate buffer as follows:

KH_2PO_4 0.0094 M	$NaHCO_3$ 0.0015 M
K_2HPO_4 0.0125 M	Sucrose 0.145 M

Specific gravity at 25°C.: 1.023. Slightly hypotonic: 0.7 X isotonic. pH 7.1.

TABLE III

Antigen Removal Tests
(Tissue + Reagent + Stain)

Reagent	Stain*			
	Conjugates		Serums	
	SCL IIb-F	DLE I-F	SCL II	DLE I
Formalin: 5% for 5 min.	+	+		
Ethanol: 95% for 10 min.	+	+	+	+
Acetone, anhydrous, 10 min.	+	+	+	+
Heating tissue 56° for 30 min.				
Wet chamber	−	−		
Wet chamber, heat conj.	−	−		
Dry chamber	+	+		
Dry chamber, heat conj.	+	+		
RNASE: 1 mg./ml. for 5 min.	+	+	+	+
DNASE:				
>0.05 mg./ml. for 1 min.	−	−	−	−
<0.01 mg./ml. for 1 min.	+	+	+	+
Trypsin:				
1 to 5 min.	+	+	+	+
5 to 15† min.	−	−	−	−
Saline buffer, 2 hr. wash before staining	+	+		

Key: + Nuclei stained
− Nuclei not stained
* Conjugates used direct; serums used indirect.
† Tissues destroyed by prolonged trypsinization.

TABLE IV

Inhibition Procedures
(Tissue + Serum + Conjugate)

Serum	Skin tissue		Kidney tissue	
	Conjugate		Conjugate	
	DLE I-F	SCL IIb-F	DLE I-F	SCL IIb-F
SCL IIa	+	−	±	±
SCL III	+	−	±	+
SCL IV	+	+	±	±
DLE I	−	+	−	+
DLE II	−	+	−	+
DLE III	+	+	−	+
DLE IV	−	+	−	+
DLE V	−	+	±	+
DMS I	+	+	+	+
Controls	−	−	−	−
Gamma globulin fractions				
SCL IIa			+	+
DLE I			−	+
DLE III			+	+

Key: + Nuclei stained
− Nuclei not stained
± Faint irregular staining

Immunologic Inhibitions. The specificity of histochemical binding was explored in a number of inhibition procedures, as represented in Table IV. In direct inhibition tests, or so-called auto-inhibitions, the tissue was treated first with an unconjugated serum, and then with the conjugate of the same serum; abolition of staining was presumed to be due to a blockade of binding sites by the raw serum. Cross inhibition procedures consisted of attempted staining of a tissue by the conjugate of a serum following prior exposure of the section to unconjugated serum from a different patient.

Microscopy

After staining, sections were inspected by ultraviolet fluorescence microscopy. Standard monocular microscopes with dark-field condensers were illuminated by automatic feed carbon arcs. Visible light was screened out by a copper sulfate cell and Corning No. 5840 filter, while stray ultraviolet was removed at the ocular by a disk of Euphos glass. Specimens were inspected at magnifications of 80, 200, and 400 times. The underside of the slide was sealed to the condenser with a drop of nonfluorescent immersion oil.

Photography

All typical reactions were recorded in both monochrome and color, using twin Leica 35 mm. cameras with Microipso attachments, the oculars of which contained Euphos filters. Black-and-white photographs were made on Kodak Tri-X film, exposed 4 to 8 minutes depending upon image intensity. These were developed commercially. For color, Anscochrome daylight emulsion was exposed 8 to 10 minutes and processed to an ASA rating of 200, rather than at the usual speed of 32. The stage coordinates for each frame were recorded, and the slide was later counterstained with hematoxylin and eosin for topographic correlation.

RESULTS

Staining Reactions

Various autologous, homologous, and heterologous tissues were stained by both the direct and indirect methods. In every case the histochemical agent was protein labeled with fluorescein isocyanate. In all 5 cases of DLE, in 4 of 6 patients with SCL, and in the single case of active DMS examined, tissue nuclei were stained intensely, fluorescing as a brilliant chartreuse green under ultraviolet light. Intranuclear localization was confirmed by subsequent hematoxylin and eosin counterstaining of the same slides, as illustrated in Figure 10.

In this report, the term "positive reaction" implies such binding of protein by intranuclear material.

The two negative cases of SCL were clinically inactive. Irregular positive staining was observed in some of the serums from patients with rheumatoid arthritis, being especially marked in those demonstrating a positive LE cell phenomenon. The reaction was negative in cases of temporal arteritis, glomerulonephritis, the case of penicillin sensitivity, and in the dozen control patients.

The direct and indirect techniques were mutually confirmatory; a given test serum was positive by both methods or not at all. Nuclei generally were stained more intensely by the indirect or layering technique. It should be noted, however, that sections stained directly showed localization only within the nuclei of tissue cells or, occasionally, in sites of focal necrosis; the background was unstained and dark, save for intrinsic autofluorescence. With the indirect technique, the final staining with anti-gamma globulin produced a generalized histochemical localization of this protein, especially in vascular lumens and extracellular interstices. Positive nuclear reactions, fortunately, were found only with serums positive by the direct stain, and never after direct anti-globulin staining alone, so that indiscriminate labeling of tissue globulin introduced no ambiguity.

Although it is customary to absorb fluorescent conjugates against desiccated tissue powder preparations, this step was omitted to prevent interference with the reactions by exposure of the reagents to intranuclear material. Indeed, binding was impaired or inhibited altogether by prior absorption. Miescher and Fauconnet,[14] it may be noted, were able to absorb the factor involved in the LE cell phenomenon, using isolated cell nuclei.

A curious inverse relation between nuclear and cytoplasmic staining was observed occasionally. In vigorously positive reactions, nuclei alone appeared to bind conjugate; with weak or negative reactions, however, a faint staining of cytoplasm was seen. Typical positive and negative reactions are shown in Figures 1 to 3 inclusive.

Effect of Tissue Fixation and Aging

Positive reactions were not prevented by fixation of the tissue in formalin, acetone, or alcohol. Although autofluorescence increased steadily with tissue aging, the nuclear substance operating in the reactions appeared stable in this respect; successful intranuclear binding has been found in sections kept at 4 to 5° C. as long as 7 days after cutting. Tissue blocks have been stored at least 18 months in a dry ice

refrigerator with no impairment of nuclear reactivity. In the present investigation, however, sections, as a rule, were stained within an hour after cutting, and were unfixed unless otherwise noted.

Correlation with the LE Cell Preparation

As shown in Table I, positive nuclear binding of gamma globulin generally paralleled positive LE preparations with serum from the same patient. The converse, however, did not hold, for a negative LE cell test did not preclude a positive reaction with nuclei.

Effect of Heat and Moisture

Heating the conjugate and tissue slices at 56° C. for 30 minutes did not prevent a subsequent positive reaction, if the tissue remained dry. Some sections were heated dry up to 65° C. for 30 minutes with no apparent ill effect. Warming in a humid atmosphere, or after direct moistening of the section, blocked the reaction.

Role of Autolyzed Protein

Frequently gamma globulin from cases of DLE, SCL, and DMS reacted with pyknotic cells or necrotic tissue constituents, with a resultant fluorescence of apparent nuclear debris. To determine whether the type of positive staining reaction under examination in the present study reflected haphazard binding with autolyzed protein, frozen sections were prepared from homotransplants of rabbit liver 12 days following the surgical procedure and treated with conjugates against both albumin and globulin. No staining whatever was observed in nuclear sites, or in the necrotic central portions of the grafts. Only the cytoplasm of peripherally disposed cells was stained, evidently because of a passive centripetal diffusion of host serum protein.

Chemical Aspects of the Nuclear Binding Reaction

Role of Serum Proteins. The preceding experimental data, particularly the results of indirect reactions, strongly suggest that the active serum principle is, or shares in the properties of, an abnormal gamma globulin. To ascertain the connection, if any, between serum protein and the reactive substances within cell nuclei, all tissues studied were surveyed topographically for albumin and gamma globulin by the use of specific antiserum conjugates. All stains were direct. Since nuclei consistently failed to be stained, they were presumed to lack significant concentrations of indigenous albumin or gamma globulin; hence the reactions probably were not mediated by the presence of these protein fractions within tissue nuclei. It is well to note that on the basis of the

indirect stain reactions performed here, as well as various protein localizations to be reported elsewhere, the anti-blood protein conjugates employed in this study appeared to stain both normal and abnormal serum proteins alike.

Role of Deoxyribonucleic Acid and Ribonucleic Acid. The participation of nucleoprotein was investigated by enzymatic digestions with specific nucleoproteinases. The staining reaction was inhibited by prior exposure of the tissue to deoxyribonuclease for 1 minute, at concentrations of 0.05 mg. per ml. or more, but not by incubation with the same enzyme for 2 minutes at 0.01 mg. per ml. or greater dilutions. At practically all concentration levels, occasional nuclei were nonreactive, but a gradually increasing inhibition was observed as the level of deoxyribonuclease was raised (Figs. 4 and 5). Ribonuclease, on the other hand, failed to prevent a positive reaction, as shown in Figure 6, even at a strength of 1.0 mg. per ml. for 5 minutes. The same buffer in which the nucleases were dissolved did not inhibit the reaction after a 2 hour contact with the tissue.

Effect of Tryptic Digestion. Crude trypsin (Viokase) was used upon sections of skin and placenta, in a dilution of 1:100, and at varying intervals up to a maximum of 15 minutes. Within the first 5 minutes of digestion, the cytoplasm of cells was destroyed, leaving behind free nuclei which nevertheless showed a bright staining reaction. The stain was abolished completely by the end of 15 minutes. In the case of skin, the cytoplasm of epithelial cells was digested away, there remaining isolated faint and shadowy remnants of nuclei, which appeared slightly autofluorescent. Placenta was destroyed almost entirely, save for scattered leukocytes which may have survived because of intrinsic antitryptic defense mechanisms. These cells appeared unaffected by trypsin and indeed were stained by the labeled abnormal gamma globulins.

Serum Inhibition Reactions

Auto-inhibition. In every instance, nuclear binding was blocked wholly when a tissue was treated first with an unconjugated abnormal gamma globulin, and then with a fluorescent conjugate of the same serum protein (Fig. 8).

Cross-inhibition. Cross-inhibition reactions were performed among the serums from different cases of DMS, DLE, and SCL. The results are presented in Table IV. Serums from lupus patients frequently, though not invariably, showed a capacity for mutual inhibition. These serums, however, generally failed to block the binding of gamma globulins from cases of SCL. The results also varied somewhat accord-

ing to the organ tested. DMS serum, as represented by the single active case studied, failed to inhibit either DLE or SCL. A typical cross-inhibition reaction is illustrated in Figure 9.

Discussion

Immediate Implications of Experimental Findings

Patients afflicted with DLE, SCL, and DMS, and also those with rheumatoid arthritis showing a positive LE cell phenomenon, appear to carry within their serums an unusual protein constituent possessing a reactive affinity with one or more intranuclear nucleoproteins in human and animal tissues. This active serum component remains with the globulin fraction after crude chemical separation, and can be tagged specifically by fluorescent conjugates against gamma globulin. It is possible to examine the abnormal globulin of such patients with the various auto- and cross-inhibition procedures employed by Coons and Kaplan[4] as measures of immunologic specificity.

The intranuclear tissue reactant which binds abnormal gamma globulins from test patients may be digested enzymatically by deoxyribonuclease solutions above a concentration level of 0.05 mg. per ml. within two minutes, while more dilute solutions appear to affect the tissue component only incompletely or not at all. Ribonuclease, in contrast, does not alter the reaction perceptibly. The receptivity of the intranuclear factor is not abolished by prior trypsinization of the tissue sections, except at concentration levels sufficient to destroy the nuclei altogether. Fixation as described does not impair the reaction.

The gamma globulin-nucleoprotein reaction is not inhibited by heating the tissue for 30 minutes at 56° C., but it is blocked, however, when the section is warmed in a moist chamber. Reactivity is not restored by the addition of fresh guinea pig serum, indicating that complement probably is not required in the *in vitro* nuclear binding phenomenon.

Since a positive reaction may be observed after heating the tissues and conjugates to 65° C. for 30 minutes, it is not likely that the combination of abnormal gamma globulin with nucleoprotein depends upon enzymatic activity.

Thus, in the collagen diseases under investigation it would seem that affected patients possess a gamma globulin which behaves at least superficially like an antibody, and hence is designated provisionally as such in this report. This putative circulating antibody binds consistently with an intranuclear substance, involving, for its reactive identity, at least DNA and perhaps also an unspecified protein moiety. The tissue reactant is not species specific, occurring in autologous, homo-

logous, and heterologous nuclear material. On the basis of available evidence, it appears to be nucleoprotein containing DNA, and may be regarded as an antigenic substance.

Histologic Correlations

The capacity of abnormal gamma globulins to react with nuclear materials, in the opinion of the authors, is the overt expression of a fundamental disturbance common to at least DLE, SCL, and DMS, and possibly other collagen diseases as well. The nuclear binding trait does not appear limited solely to those patients with positive LE reactions; cases of SCL, among others with negative LE cell preparations, exhibited positive nuclear staining responses.

In all probability the LE cell inclusions and the basophilic bodies commonly found in DLE are interrelated phenomena. On the basis of histochemical evidence, Klemperer, Gueft, Lee, Leuchtenberger and Pollister[15] regarded the two structures as one and the same, and the finding of DNA in both has made their nuclear origin indisputable.

Alteration of the nucleus appears prerequisite to the development of the LE cell inclusions and basophilic bodies, as was demonstrated *in vitro* by Snapper and Nathan[16] in a case of DLE; little imagination is necessary to extend the same factitive process to the basophilic bodies as well. The interrelationship is strengthened further by the fact that the formation of both structures is mediated through the gamma globulin serum fraction of affected patients. Haserick and his colleagues[3] were able to duplicate the LE cell phenomenon with serums free of gamma globulin, while Gueft[17] reported the presence of unusually large amounts of protein within LE cell inclusions. Mellors, Ortega and associates[18,19] have localized gamma globulin in the inclusions by means of the fluorescent antibody technique. It may be concluded, therefore, that nuclear alteration or the formation of basophilic bodies is the initial step in a developmental sequence; this is followed by cytophagocytosis resulting in LE cells. Since both nuclei and serum gamma globulin are involved in the formation of basophilic bodies and LE cells, these agents probably also participate in the nuclear binding trait reported here; the latter effect thus may represent a broad and fundamental phenomenon related to the pathogenesis of the several diseases under investigation.

Clinicopathologic Correlations

The literature devoted to DLE is extensive, and only a few key references need be cited.[20-22] The salient clinicopathologic features have been summarized by Dubois.[23] Available information on SCL is

increasing rapidly,[24-28] and DMS, the least common of the three, has been discussed at length by Keil[29,30] and by Kinney and Maher.[31]

The question of whether DLE, SCL, and DMS reflect one and the same disease process has long been the basis for disagreement. Indications of similarities and differences have been as numerous as the authors proposing them. Recently Kampmeier[32] has submitted examples of interconversion in support of a hypothesis that all of the so-called collagen diseases are inherently interrelated. On purely clinical grounds, Beigelman, Goldner and Bayles[33] have classified DLE and SCL as expressions of a common disease mechanism which, when acute and severe, is seen as DLE, and when chronic and benign, appears as SCL. The classification of acrosclerosis is also controversial.[24,34] Because the single case studied in the present survey gave strong and reproducible nuclear binding reactions, the authors believe this curious disease may be considered a variant of SCL.

As a result of the experimental findings described here, as well as other work still in progress, it appears likely that the nuclear binding trait may reflect a natural phenomenon which is genuine, basic, and common to DLE, SCL (including acrosclerosis), DMS, and possibly a number of other diseases as well. The latter would include those cases of rheumatoid arthritis accompanied by positive LE preparations. Should these several clinical forms not be identical, they nevertheless appear to share similar properties, at least in relation to a demonstrable reactive affinity between serum gamma globulins and DNA-protein.

The binding trait can be blocked by auto-inhibition, and—at times—by cross-inhibition. The former tests were consistent, but the latter gave variable responses. In any consideration of inhibition experiments, it is well to bear in mind the reversibility of antigen-antibody combinations. The matter of concentration, not investigated thoroughly at this time, is also of great importance. It is noteworthy that a typical positive reacting serum from SCL, SCL IIb, which by itself gave constantly intense reactions, regularly failed to cross-inhibit a weaker conjugate from DLE, DLE I-F.

From the results of the various inhibition tests, one may conclude that sensitization in one case may be due to one or more DNA-proteins, while in another patient the responsible tissue reactant may be one or more antigenically distinct DNA-proteins. Regardless of the initiating agent, the common end result is the same fundamental pathologic sequence.

Pathogenetic Considerations

Since the publication of the concepts concerning connective tissue disorders by Klinge[1] and Klemperer,[2] and the inclusion of SCL in this group by Masugi and Yä-Shu,[35] these disorders have been ascribed

generally to some sort of allergic state. A basis in the immunity mechanism, however, was supported principally by superficial morphologic considerations, such as the nonspecific features of fibrinoid degeneration and hyalinization of connective tissue. Despite many other theories,[36-41] hypersensitivity remains in broadest favor, even though supporting evidence in the literature has been only fragmentary.[18,19,42,43]

In addition to the auto- and cross-inhibition tests reported here, the results of animal experimentation offer strong, although not absolute, evidence in support of a hyperimmune mechanism. In this laboratory, following the active immunization of albino rabbits against calf thymus nucleoprotein, the serum gamma globulin from these animals exhibited the same type of histochemical reaction seen in our clinical cases. There was binding not only with calf thymus nuclei, but also with the nuclei of other autologous, homologous, and heterologous tissues. The experimentally produced rabbit antiserum against nucleoprotein, moreover, showed not only auto-inhibition but also positive cross-inhibition with the serums of some patients. Details of the animal series, and of various confirmatory *in vitro* procedures, will be furnished in a subsequent report.*

It is appropriate to inquire in what manner sensitization may occur initially. No concrete answer can be offered at the present time. Relying, however, upon the experimental evidence of non-species specificity in the gamma globulin-nucleoprotein binding reaction, it is suggested that sensitization, whether immunologic or not, may begin as a response against heterologous nucleoproteins, becoming manifest thereafter as autoimmunity against one or more native intranuclear substances.

One possible pathway for the development of the disease process may be through intra-uterine sensitization against fetal or placental tissues. The first case investigated, SCL I, did not show positive nuclear staining until an episode of acute eclampsia. Indeed, this patient has related temporally her various medical problems, including SCL, to the onset of eclamptic seizures, claiming good health before that time. SCL in this patient has remained clinically active during the 18 months she has been followed, and her serum specimens have presented

* Since the preparation of the present manuscript, a pilot study has indicated that the histochemical phenomena described here may be duplicated *in vivo* by techniques based upon the general premise of hypersensitivity against nucleoprotein, suggesting the possibility of eventual production of the disease complex by artificial means. Rabbit anti-histone antiserum has exhibited identical nuclear staining capacity, as well as cross inhibition with serum from a case of human scleroderma. A preliminary report has been published elsewhere (Bardawil, W. A.; Toy, B. L., and Galins, N. Hypersensitivity to histone induced experimentally in rabbits. [Preliminary communication.] *Lancet*, 1958, 1, 888–889). These findings have been confirmed by a subsequent investigation currently in progress; further details will be reported in the near future.

strong and constant positive nuclear reactions during this period.* The problem of intra-uterine sensitization in animals has been explored by Brambell, Hemmings and Henderson.[44]

The apparent sequential association between collagen disease and antecedent streptococcal infections is well known, albeit poorly understood. Swineford and Holman,[45] in 1949, reported a tendency to delayed local reactions following immunization with crude nucleoprotein extracts from a number of bacterial species, including both *Streptococcus viridans* and *Streptococcus hemolyticus*. Bacterial or viral DNA-protein, derived from invading micro-organisms, merits inclusion in a list of hypothetical heterologous antigens.

Once established, a state of sensitivity may be maintained by the small amounts of nucleoprotein liberated as cells perish spontaneously, as in physiologic necrobiosis, or are destroyed by pathologic events. Bunting[46] has described the interstitial accumulation of deoxyribonucleoprotein in areas of necrosis within a number of organs. Billingham, Brent and Medawar[47] have presented evidence that, at least in the case of reactions to skin transplantation, nucleoprotein hypersensitivity appears to be a significant factor. The same investigators have suggested that the release of nucleoprotein from living cells in the body may be a normal and constant occurrence.

Trauma may play an important role in the pathogenesis of certain lesions in a given disease, very much in the manner proposed by Massell, Mote and Jones[48,49] in the formation of subcutaneous nodules in rheumatic fever. The possible implication of some type of enzymatic activity has been studied by Schlamowitz, DeGraff, Schubert and Morrison[50-53] in connection with rheumatoid arthritis.

The elucidation of what, if any, role is played by the establishment, maintenance, and interruption of nucleoprotein hypersensitivity in the clinical course of patients affected with DLE, SCL, and DMS must await the results of further inquiry. In fact, a better understanding of the natural history of the DLE-SCL-DMS complex will follow upon improved knowledge of nucleoprotein metabolism in both normal and deranged states.

Summary

A peculiar binding trait of gamma globulin has been observed in patients with disseminated lupus erythematosus, scleroderma, and dermatomyositis. By the use of fluorescent antibody techniques, this serum factor has been shown to possess a reactive affinity with intranuclear material, probably deoxyribonucleoprotein, within autologous, homologous, and heterologous tissues. The findings are correlated and

* This patient has died 24 months after the onset of disease, with necropsy findings typical of generalized scleroderma.

prompt the suspicion that the above diseases, and perhaps related conditions such as rheumatoid arthritis, may be manifestations of a common disease process initiated by sensitization against either intrinsic or extrinsic nucleoprotein. Nuclear binding appears to require the participation of DNA but not RNA, and complement probably is not involved in the *in vitro* phenomenon. The pathogenesis of so-called diseases of collagen, as represented by those investigated, is discussed briefly.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Figures 1 to 9 inclusive photographed by ultraviolet light in fluorescence microscope, as described in text.

FIG. 1. Homologous positive nuclear staining of normal immature human placenta by conjugate from case SCL Ia-F. Direct technique. × 100.

FIG. 2. Heterologous positive staining of normal rabbit liver by serum from case SCL II, followed by anti-human gamma globulin conjugate AHG. Indirect technique. × 260.

FIG. 3. Typical negative nuclear reaction, by indirect technique. Normal human placenta, stained by anti-human gamma globulin conjugate AHG. × 260.

FIG. 4. Digestion of normal human placenta by deoxyribonuclease at concentration of 1.0 mg. per ml. for 3 minutes, with subsequent indirect stain by serum from case SCL II, followed by conjugate AHG. Note inhibition of nuclear binding reaction. × 260.

FIG. 5. Digestion of normal human skin by deoxyribonuclease at concentration of 0.01 mg. per ml. for 2 minutes, followed by direct stain with conjugate DLE I-F. Note failure to inhibit reaction. × 100.

FIG. 6. Normal immature human placenta, stained with serum from case SCL II, followed by conjugate AHG, after prior digestion by ribonuclease for 5 minutes at a concentration of 1.0 mg. per ml. Note failure of ribonuclease to inhibit positive nuclear binding reaction. × 260.

FIG. 7. Homologous positive nuclear staining of normal human kidney by conjugate SCL IIb-F. Direct technique. × 100.

FIG. 8. Abolition of nuclear binding reaction by auto-inhibition. Normal human kidney stained with serum from case SCL II, followed by conjugate SCL IIb-F from the same specimen. × 100.

FIG. 9. Failure of cross-inhibition to abolish nuclear binding. Normal human kidney stained with serum from case DLE I, followed by conjugate SCL IIb-F. Note weak staining of nuclei in glomerulus. × 100.

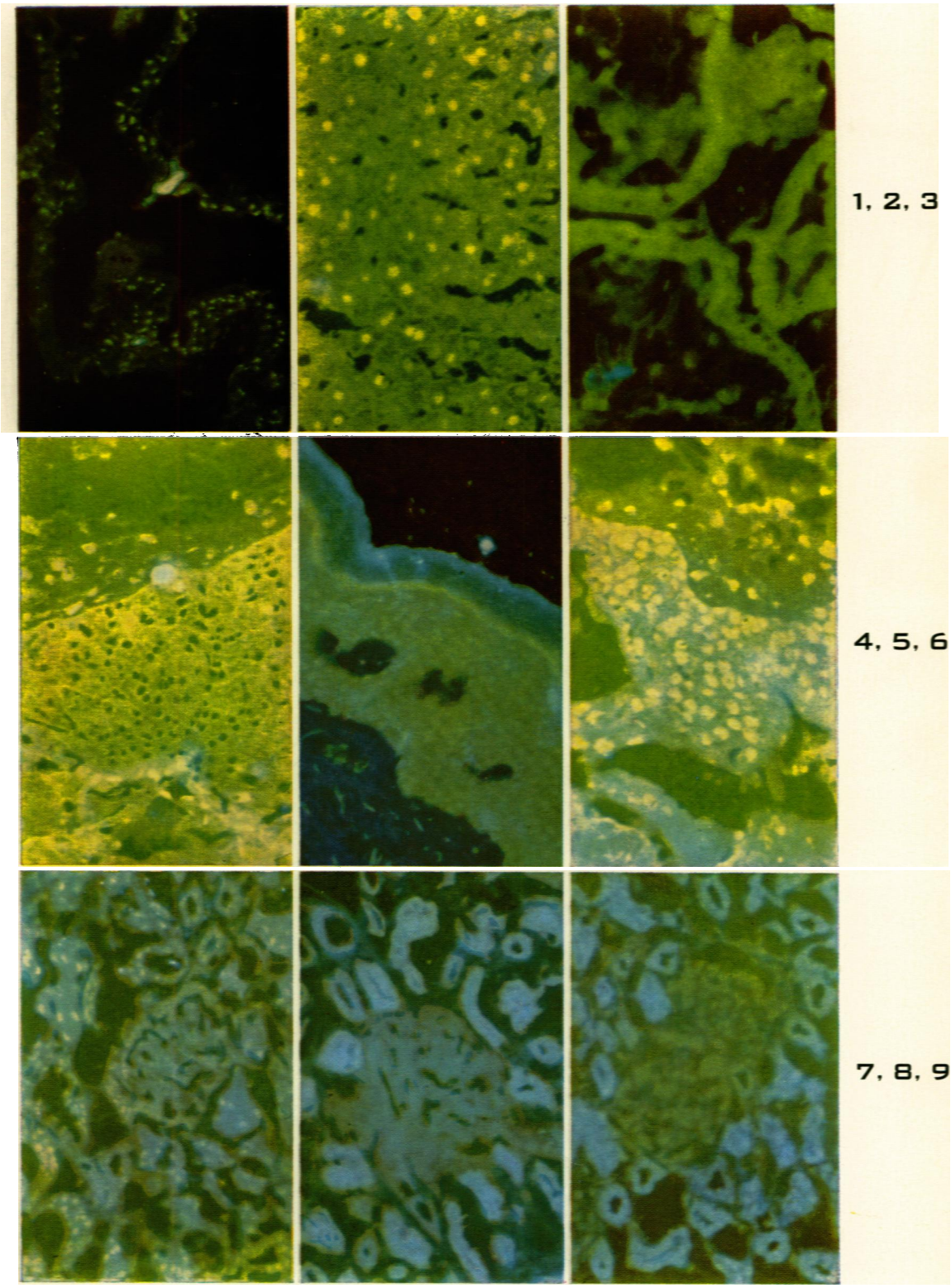
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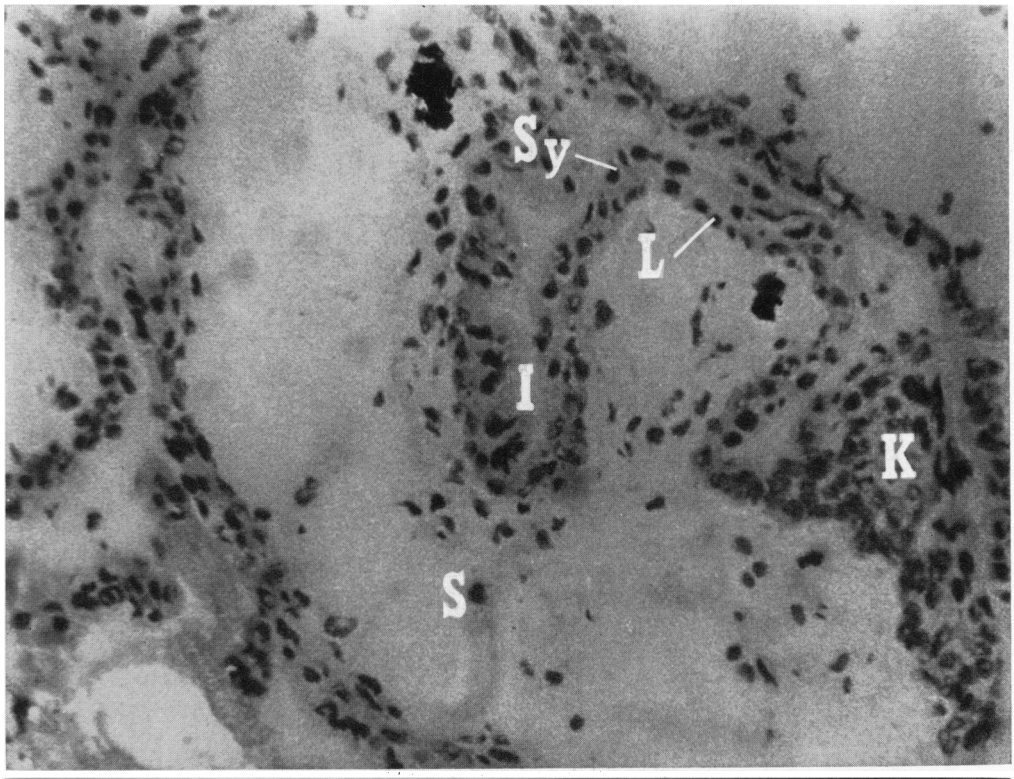
FIG. 10. Normal immature human placenta, showing general topography of a single villus in cross section. Frozen section, stained with hematoxylin and eosin and photographed by visible light. I, intervillous space; K, syncytial knot; L, Langhans layer; S, stroma in core of villus; Sy, syncytial trophoblast. × 260.

FIG. 11. Same section as shown in Figure 10, after direct staining with conjugate SCL Ia-F. Note positive nuclear binding reaction, verified by counterstain in Figure 10. Photographed by ultraviolet light. × 260.



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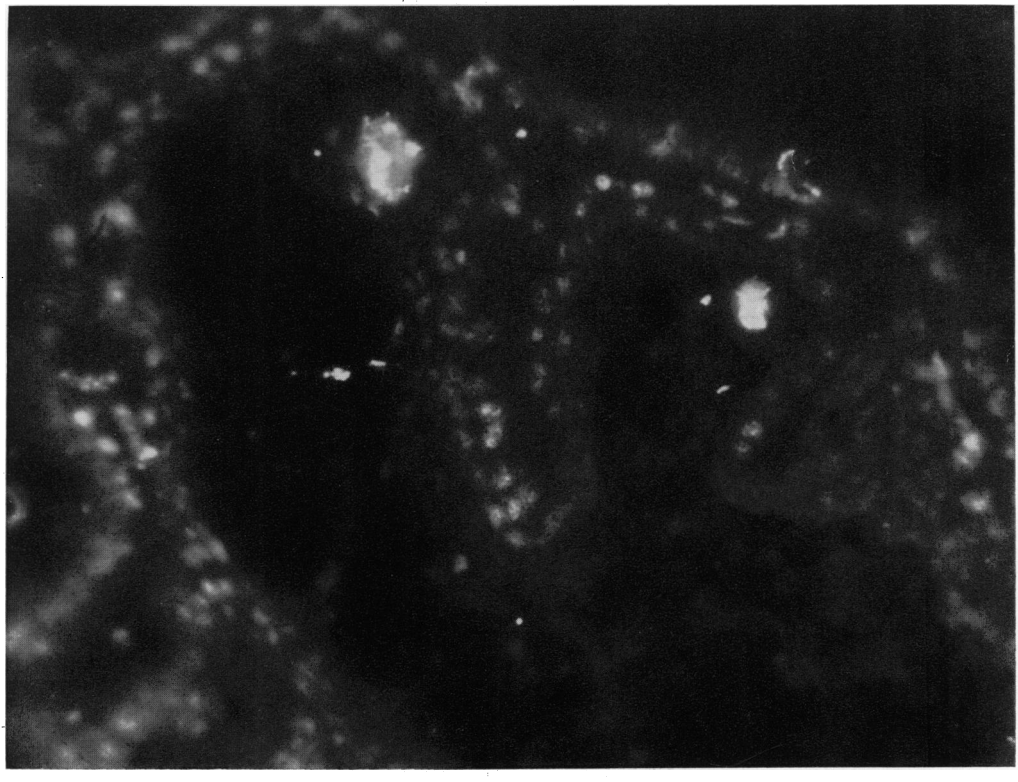

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