NUCLEAR FLUORESCENCE EMPLOYING ANTINUCLEOSIDE IMMUNOGLOBULINS*

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Plates 1–4

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The sensitive technique of immunofluorescence (1) has been used widely to demonstrate a nuclear reaction with serum globulins of patients with systemic lupus erythematosus (SLE) (2–5). These sera have multiple specificities to deoxyribonucleic acid (DNA), protein, DNA-protein, and carbohydrate (6–9)) and until recently, were the only source of DNA-reactive antibodies. The stimulus for their formation remains unknown.

It is the purpose of this paper to report a specific nuclear reaction employing fluoresceinated conjugates of DNA-reactive antibodies produced in response to immunization with nucleosides coupled to bovine serum albumin (BSA) (10). Unlike SLE sera, the antibodies employed in this study have been shown previously to be highly base specific, capable of reacting exclusively with denatured DNA and are produced in response to the injection of a chemically characterized antigen. The experiments reported herein were intended to provide information which might serve as a guide in the design of experiments with these antibodies on living systems.

Methods

Antigens and Immunization.—The preparation of the nucleoside conjugates, the protocol of immunization, and the schedule and manner of bleeding have been described previously (10). Both sheep and rabbits were immunized with BSA conjugates of the five major nucleic acid bases and BSA itself (adenosine (A), cytidine (C), guanosine (G), uridine (U), and 1β-D ribofuranosyl thymine (T) which was used instead of thymidine (10)). Conjugates are abbreviated A-BSA, U-BSA, etc.

Antibody Testing.—Antisera were tested by double diffusion in agar (11) or by the quantitative precipitin method (12).

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Fluorescein Conjugates.—Fluorescent sheep anti–rabbit globulin and fluorescent rabbit anti–sheep globulin were obtained from Pentex Co., Kankakee, Ill. and from Nutritional Biochemicals Corp., Cleveland, Ohio. Using antinucleoside or anti–BSA antisera, globulin preparation and purification of the fluoresceinated conjugates were carried out as described by Dedmon, Holmes, and Deinhardt (13). Crystalline fluorescein isothiocyanate (Sylvana Chemical Company, Orange, N. J.) was conjugated to protein following the procedure of Hsu (K. Hsu, personal communication). After each step in the preparation and purification of conjugates, the antisera were checked for the presence of antibody by the gel diffusion method.

Tissue Culture.—Mouse L cells (derived from strain 929, Earle, and donated by Dr. C. Howe of this department) were grown in 250-ml plastic tissue culture flasks (Falcon Plastic Company, Los Angeles, Calif.) at 37°C for 4–7 days. The sheet was detached and dispersed in 0.25% trypsin in phosphate-buffered saline with sodium ethylene diamine tetraacetate and glucose (14).

The cells were collected by centrifugation and suspended in culture medium. (Eagle's minimum essential medium (15) with 10% fetal calf serum, heat treated at 56°C for 30 min to inactivate possible mycoplasma contaminants; penicillin G, 15 units/ml; streptomycin, 15 mcg/ml; double the concentration of glutamine, cofactors, and vitamins; and nonessential amino acids at 0.1 mM: L-alanine, L-asparagine·H_2O, L-aspartic acid, L-glutamic acid, L-proline, L-serine, and glycine. All medium constituents were obtained from Grand Island Biological Company, Grand Island, N.Y.)

Plastic flasks with 25 ml culture medium were inoculated with 4×10^6 cells (approximately) or Leighton tubes containing cover slips were inoculated with $1.3–1.5 \times 10^5$ cells in 1 cc medium. The cover slips were removed after various times of incubation (see Results) and, after three washes of 5 min each in phosphate buffer saline (PBS) (0.14 M NaCl 0.01 M PO_4 pH 7.3), were fixed by one of the following methods: (*a*) air-drying 1–2 hr at 37°C; (*b*) air-drying 1–2 hr at 37°C, storing at 4°C for 1–21 days, and dipping in methanol at room temperature immediately prior to staining; and (*c*) air-drying 1–2 hr at 37°C, placing at 4°C for 1–21 days followed by treatment with 95% ethanol for 30 sec at room temperature just prior to staining.

Fluorescent Antibody Staining.—Antisera were diluted in PBS to 2–4 mg/cc total protein as measured by a hand refractometer. The cover slips were placed on a small staining rack in a humidity chamber and 5 drops of antiserum were added onto the surface of each cover slip. The antiserum was allowed to react for 20 or 30 min at room temperature. Then the cover slips were drained and rinsed with a forced stream of PBS. This was followed by 2 washes in PBS of 10 and 5 min duration and another vigorous rinsing with PBS. The cover slips were then mounted in glycerol (1 part): PBS (4 parts) pH 7.5 on standard microscope slides (less than 1 mm thick) and immediately examined under the UV microscope.

In "blocking" experiments, the unfluoresceinated globulin was allowed to react with the cells as above for 1 hr (1 replacement with fresh globulin at ½ hr) at 37°C. The cover slips were washed (see above) and then stained with the fluoresceinated antiserum.

Cells treated with deoxyribonuclease (DNase) (16) (Worthington Biochemical Corp., Freehold, N.J.) were stained with fluorescent antibody. The control consisted of cover slips treated with heat-inactivated (56°C, 40 min) enzyme, then stained.

The distribution of DNA within the cell, particularly the nucleus, was examined by staining using the Feulgen reaction (17). In the Feulgen reaction, hydrolysis was carried out for 15 min at 60°C. The reaction of the cells with the Schiff reagent was allowed to proceed in the dark. For control slides the acid hydrolysis was omitted.

Thymidine Uptake.—An accurate quantitation of the thymidine uptake per L cell requires either that a constant number of cells be used or that an accurate estimate of the number of cells be possible. Since it was not possible to distribute the cells in a reproducible manner, a means of counting them had to be devised. For this purpose, 10 μl thymidine-^{3}H (thymidine-

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methyl-^{3}H, 0.25 mc in 0.5 ml, 3.0 c/mmole, lot 3505 Schwarz Bio Research, Inc., Orangeburg, N.Y.) were added to the media of a single fresh subculture of L cells, and the culture was allowed to grow to stationary phase. 5 days after the original subculture, the medium (25 ml) was removed and the culture washed 3 times with 10 cc of nonradioactive medium. Then 25 ml of fresh medium were added, and the culture was trypsinized 6–8 hr later. The purpose of this procedure was to incorporate thymidine-^{3}H into the DNA of the cells so that the number of counts from tritium would be proportional to the number of cells. A suspension of 1.3–1.5 $\times$ 10^5 tritiated cells/ml was divided into two equal parts (A, B). 1 ml aliquots of A were distributed into Leighton tubes. To B, thymidine-^{14}C (0.05 ml per ml of cell suspension · thymidine-2-^{14}C, 100 μc in 10 ml, 26 μc/mmole, lot 6507, Schwarz Bio Research, Inc.) was added. Then 1 ml aliquots of B were distributed into Leighton tubes. The Leighton tubes were incubated at 37°C for various times (see Results) and at each interval cover slips were removed from groups A and B. Cover slips from A were washed and fixed for staining with fluorescent antibody. B cover slips were washed twice for 5 min each in PBS at 4°C, then placed in 5% trichloroacetic acid for 30 min followed by 2 washes of 10 min duration. The cover slips were air-dried, placed in scintillation vials, and counted in a liquid scintillation counter. Thymidine uptake per unit of cells was calculated by using the ^{14}C counts per minute (cpm) to ^{3}H cpm ratio.

Microscopy and Photography.—A Zeiss Standard Universal Microscope fitted with an HBO 200 Osram Mercury burner and a 1.2/1.4Z dark field condenser was employed for UV microscopy. The exciter filters were BG 12, 4 mm, and BG 38, 2.5 mm. Barrier filters were Zeiss 47 or 50. The lens system consisted of a 40 $\times$ oil immersion objective, a 10 $\times$ eyepiece (5 $\times$ eyepiece to camera) and a 2 $\times$ optovar. Photographs were taken with a Zeiss Ikon 35 mm camera (factor 0.5) using Anscochrome 200 ASA color daylight or Agfa Isopan Record (black and white) film.

RESULTS

Fluoresceinated antisera to A, G, T, and U all were capable of producing nuclear fluorescence in ethanol-(or methanol-) fixed L cells. (Anti-C has been prepared but not tested.) The pattern of immunofluorescence (Figs. 1, 2) was clumped and threadlike, remained unchanged over 2–3 dilutions of antiserum, and occurred in almost all the cells on the cover slips. Minimal but repeated fluorescence of the cytoplasm was observed also (Fig. 1). Staining of nucleoli was variable. On occasion the nucleoli were completely unstained and stood out prominently against the nuclear fluorescence (Fig. 3), while in other instances there was definite nucleolar fluorescence (Fig. 4). This was also true of the staining of the nuclear membrane (Figs. 2–4).

The pattern of nuclear fluorescence was strikingly similar to the distribution of nuclear DNA as shown by staining with the Feulgen reaction (Fig. 5).

The specificity of the nuclear reaction of the antisera was demonstrated by the following controls: (*a*) Absorption (with A-BSA) of the specific antibody from an antiadenosine globulin eliminated all fluorescence. (*b*) Treatment of the cells with nonfluorescent antiadenosine globulin followed by staining with the fluorescent globulin ("blocking" experiments) eliminated almost all of the fluorescence, of the nucleus, nuclear membrane, and nucleoli. The cytoplasmic fluorescence, while faint to begin with, did not seem to be affected by this procedure (Fig. 6). (*c*) Treatment of cells with a solution of DNase destroyed the

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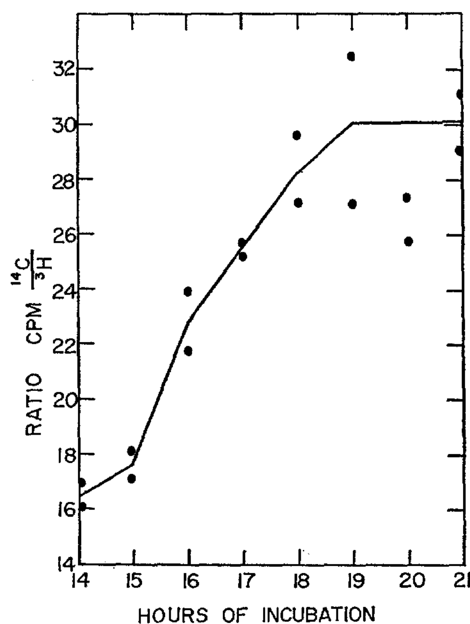
ability of the nucleus to combine with anti-U fluorescent antibodies (Fig. 7). It did however produce very bright cytoplasmic staining. Control slides treated with heat-inactivated enzyme showed nuclear fluorescence comparable to that of cells reacted with anti-U alone. (*d*) No nuclear fluorescence was observed in ethanol-fixed cells (see Methods), using fluoresceinated anti-BSA, although diffuse staining of the cytoplasm did occur (Fig. 8). (If, however, the cells were



TEXT-FIG. 1. Thymidine uptake by L cells as a function of time of incubation (see Methods for details).

fixed in methanol, nuclear fluorescence did occur. Certain commercial fluoresceinated antiglobulins (see Methods) also gave nuclear fluorescence when reacted with methanol-fixed cells.)

During the course of this work, it became apparent that the ability of the cells to react with the antisera was dependent upon the length of time cells were incubated prior to staining. L cells incubated 16–18 hr after subculture showed nuclear fluorescence as well as the nucleolar, nuclear membrane, and faint cytoplasmic fluorescence mentioned previously. However, incubation for 9, 10, 12, 14, 20, 21, 22, 25, 34, and 42 hr yielded cells in which, with one exception, only cytoplasmic staining occurred. At 20 hr, in one preparation, nuclear membrane fluorescence was observed. The relationship of the 16–18 hr period to DNA synthesis and mitosis was therefore explored. From the mitotic index

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(2–3%) of cells during this period, information about the lag period, and the time of the first expected mitosis, it was clear that the 16–18 hr period preceded mitosis. The data from the thymidine uptake experiments (Text-fig. 1) indicate that it was during the 16–18 hr period that the highest rate of thymidine incorporation took place. Text-fig. 1 illustrates a low rate of thymidine incorporation between 14–15 hr and 19–21 hr of incubation. However, beginning at 15 hr and ending at 19 hr, the average rate of thymidine uptake is more than 3 times the rate before or after this period.

DISCUSSION

Evidence has been presented that antinucleoside antibodies react with cell nuclei, nucleoli, and nuclear membranes in a specific manner. Furthermore, the ability of the cells to so react is limited to a specific stage in their growth cycle. This stage coincides with DNA synthesis by the cells.

The antibodies used in this study were of known specificity (10), obtained by injecting a chemically defined antigen. Although Cohn fraction V (BSA) is not chemically pure, immunization with this alone failed to produce DNA reactive antibodies (unpublished observations and reference 18). Thus, the actual antigen responsible for the production of anti-DNA antibodies, the nucleoside conjugated to BSA, is a well defined if not homogeneous substance (i.e. the degree of conjugation may vary from one molecule to another). Furthermore, the antibodies react exclusively with denatured, rather than native DNA, and their specificity has been characterized by hapten-inhibition.

The pattern of nuclear fluorescence obtained in this study was different from that observed by Seligmann (using leukocytes) (4), but closely resembled the localization of fluorescence noted by Rapp (5), (cf. Fig. 4). The type of pattern may be a function of the fixation techniques and cell types (leukocytes, fibroblasts, etc.) used. In the present study and in the work of Rapp, air-dried tissue culture cells grown on cover slips were used. Ethanol or methanol was used primarily to cause the cells to adhere to the cover slip, since air-drying alone proved unsatisfactory.

Specificity of the nuclear reaction was indicated by the results of control experiments (see Results). The specificity of the cytoplasmic fluorescence, however, is open to discussion. No nuclear fluorescence occurred in experiments using absorbed antiserum; cytoplasmic staining was also absent. No nuclear fluorescence was observed in "blocking" experiments using nonfluorescent antinucleoside globulin followed by a fluoresceinated preparation of the same globulin. Slight cytoplasmic fluorescence did occur. Nuclear fluorescence was eliminated by treatment of the cells with DNase prior to staining. However, marked cytoplasmic fluorescence was observed. This may have been the result of release of oligonucleotides from the nucleus into the cytoplasm.

It is not possible to ascribe a definite significance to the faint cytoplasmic

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staining observed in all experiments in which cells were exposed to fluoresceinated antinucleoside antibody. On the other hand, removal of specific antibodies from the serum by absorption eliminated both cytoplasmic and nuclear fluorescence, suggesting that cytoplasmic staining is not a nonspecific effect. Membrane-bound nucleotides, ribosomes, or cytoplasmic DNA are extranuclear structures that might be implicated in the cytoplasmic staining.

It is concluded from the DNase sensitivity of the nuclear staining and from the distribution of Feulgen-positive material in the nucleus that the nuclear reaction is dependent on DNA. The presence of nucleolar fluorescence raises the question of the presence of DNA in nucleoli or of a reaction with ribonucleic acid (RNA). The latter seems more likely in view of Sela's finding (19) that his uridine-specific antibody will react with RNA.

To demonstrate the specificity of the nuclear reaction further, antibodies to BSA were prepared and fluoresceinated. BSA was chosen because it was the carrier for the nucleosides used for immunization. Since fetal calf serum was present in the tissue culture medium, it was expected that cytoplasmic fluorescence would be observed. This, indeed, was found if the cells were air-dried and fixed in ethanol, but nuclear fluorescence was not observed.

The rather strict time-dependence of nuclear staining would seem to be of considerable significance. In past studies it has been shown that antinucleoside antibodies will react with denatured, but not with native DNA. Therefore, the fact that the antibodies react with DNase-sensitive substances in the cell nuclei (distributed similarly to Feulgen-positive materials) at a time when DNA is replicating, suggests further that the reaction is with DNA and that this DNA is at least partially single-stranded. This also points to a potential biological role for antinucleoside antibodies, assuming that they can gain entrance into the cell; they might interfere with DNA synthesis. Such studies are currently being pursued in this laboratory using protoplasts and suspension cell cultures.

The experiments with thymidine suggest that, although no attempt was made to obtain a synchronous culture, there was a high degree of synchrony as measured by the magnitude of the slope of the thymidine incorporation curve. This degree of synchrony accounts for the high percentage of cells that exhibit nuclear staining.

Many investigators have shown that globulins from patients with SLE react with DNA and with cell nuclei. Anti-DNA and/or antinuclear antibodies have also been detected after immunization with human serum (18) and with bacteria (20). The stimulus for the antibody production in both instances is unknown. Seligmann (4), Beck (7, 21), and Lachman (9) have described various patterns of immunofluorescence caused by SLE sera and have attempted to relate this to the stimulus which elicited these globulins. The membranous pattern has been correlated with anti-DNA. A homogeneous pattern of nuclear immunofluorescence has been attributed to reaction with nucleoprotein whereas

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the "speckled" reaction is thought not to involve DNA or DNA-protein. To date the work demonstrating nuclear immunofluorescence has been performed with sera which reacted with nucleoprotein, calf thymus cell nuclei, or native and denatured DNA (4, 6). Reviews (6, 21–23) on this subject do not mention a work in which nuclear immunofluorescence has been produced with a sera of sharply defined specificity, reactive only with denatured DNA, and produced in response to injection of a single antigen.

Although the formation of circulating antinuclear and anti–DNA antibodies has not been shown to play a pathogenetic role in experimental animals, there is evidence to suggest that eliminating anti–DNA antibodies from pathogenetic considerations in SLE is premature (21, 24–28). Failure to demonstrate cytotoxicity or a reaction with antibodies directed against internally located antigens using standard tissue culture techniques needs no discussion (5, 21, 29–32). This may only prove that the techniques used were not suitable. Using a different system, Rosenkranz, *et al.* (33), were able to arrest division in fertilized sea urchin eggs with various purine and pyrimidine specific antisera. However, a biological role of anti–DNA antibodies still remains to be demonstrated clearly.

Regarding use in vitro, these antibodies would seem to be an excellent tool to study distribution of nucleic acids within cells, to follow virus multiplication and perhaps to detect rapidly multiplying cells. Our further studies will bear on biological effects of these antibodies on growth, differentiation, and multiplication of living cells and the possible pathological effects of long-term immunization with antigens which elicit such antibodies.

SUMMARY

Fluoresceinated antinucleoside globulins were shown to react with the nuclei of L cells. The pattern of nuclear fluorescence was similar to the distribution of nuclear DNA. This reaction was shown to be specific by the following control experiments:

1. Absorption of the specific antibody from an antiadenosine globulin eliminated all fluorescence.

2. Treatment of the cells with nonfluorescent antiadenosine globulin followed by staining with the fluorescent antiadenosine eliminated almost all of the fluorescence of the nucleus.

3. Treatment of the cells with DNase destroyed the ability of the nucleus to react with antiuridine fluorescent antibodies.

4. Fluoresceinated anti-BSA did not produce nuclear fluorescence.

Nuclear fluorescence occurred only in cells harvested during the period of maximum DNA synthesis as measured by the uptake of thymidine. This correlates with the previously demonstrated specificity of the antibodies for denatured DNA.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Fluorescence of L cell nuclei using fluorescein-conjugated sheep anti-U. × 2400.

Fig. 2. Fluorescence of L cell nuclei using fluorescein-conjugated rabbit anti-A. × 2400.

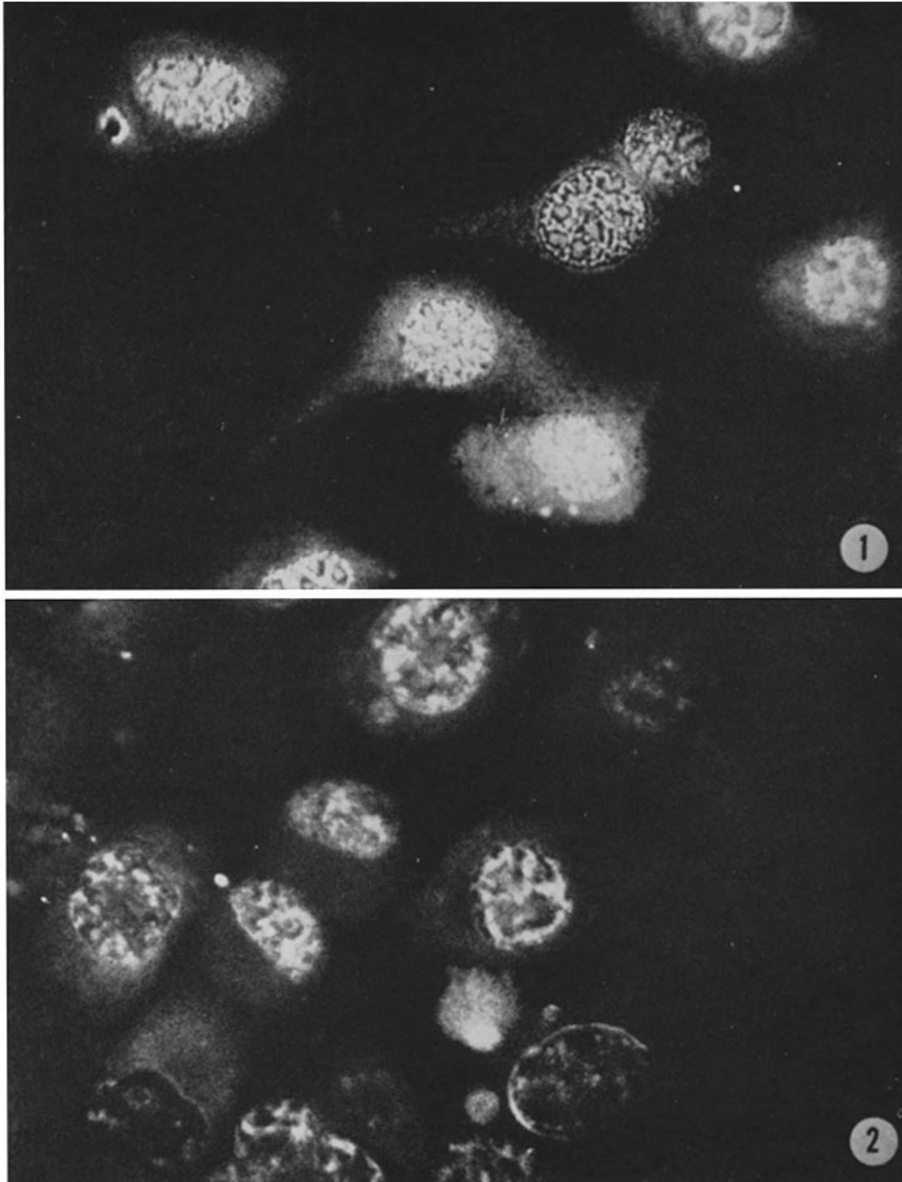

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Plate 2

Fig. 3. Fluorescence of L cell nuclei using fluorescein-conjugated sheep anti-U. Note absence of nucleolar and nuclear membrane fluorescence (arrow). $\times$ 2400.

Fig. 4. Fluorescence of L cell nuclei using fluorescein-conjugated sheep anti-U. Note fluorescence of nucleoli and nuclear membranes (arrows). $\times$ 2400.

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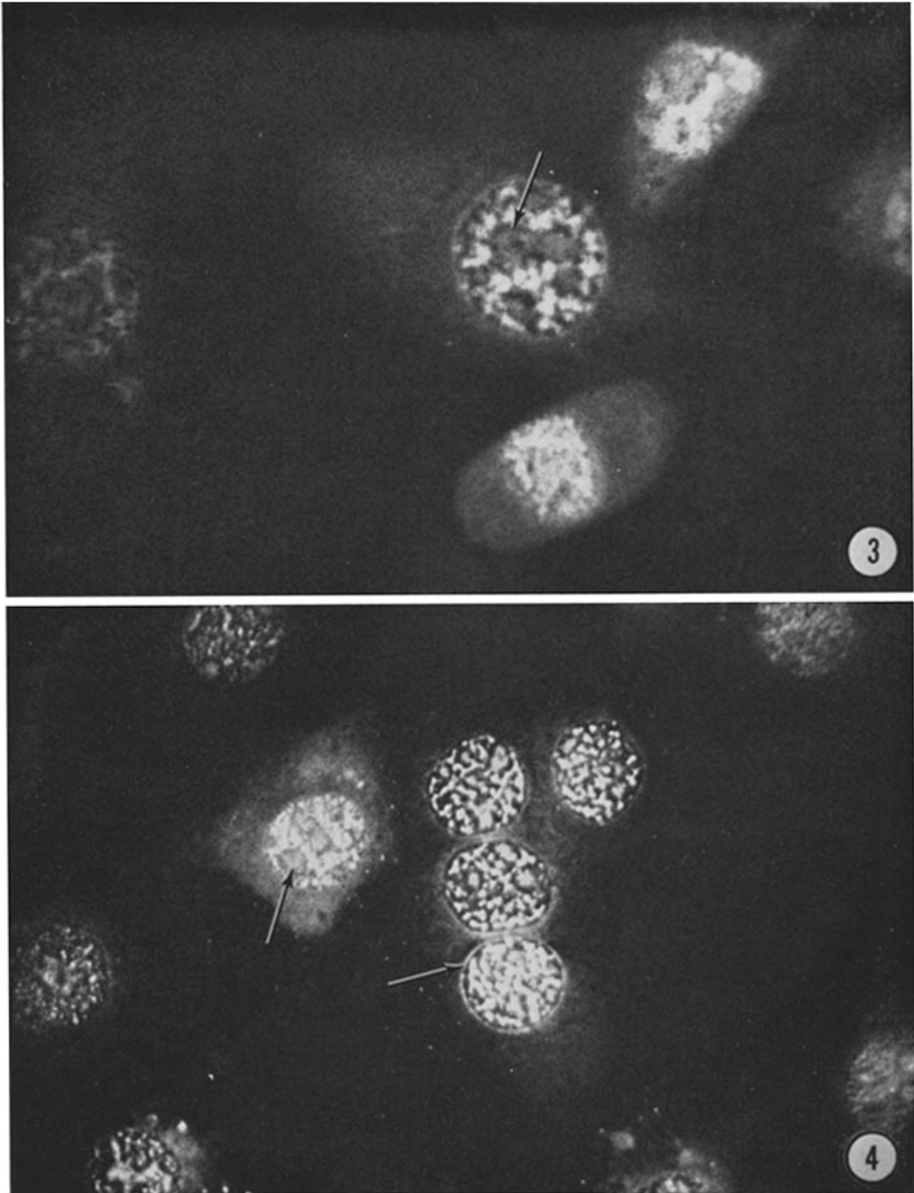

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Plate 3

Fig. 5. L cells stained using Feulgen reaction to demonstrate pattern of DNA distribution. × 2400.

Fig. 6. Marked reduction in nuclear, nuclear membrane, and nucleolar fluorescence observed in "blocking" experiments (nonfluorescent anti-A followed by staining with fluoresceinated anti-A). × 2400.

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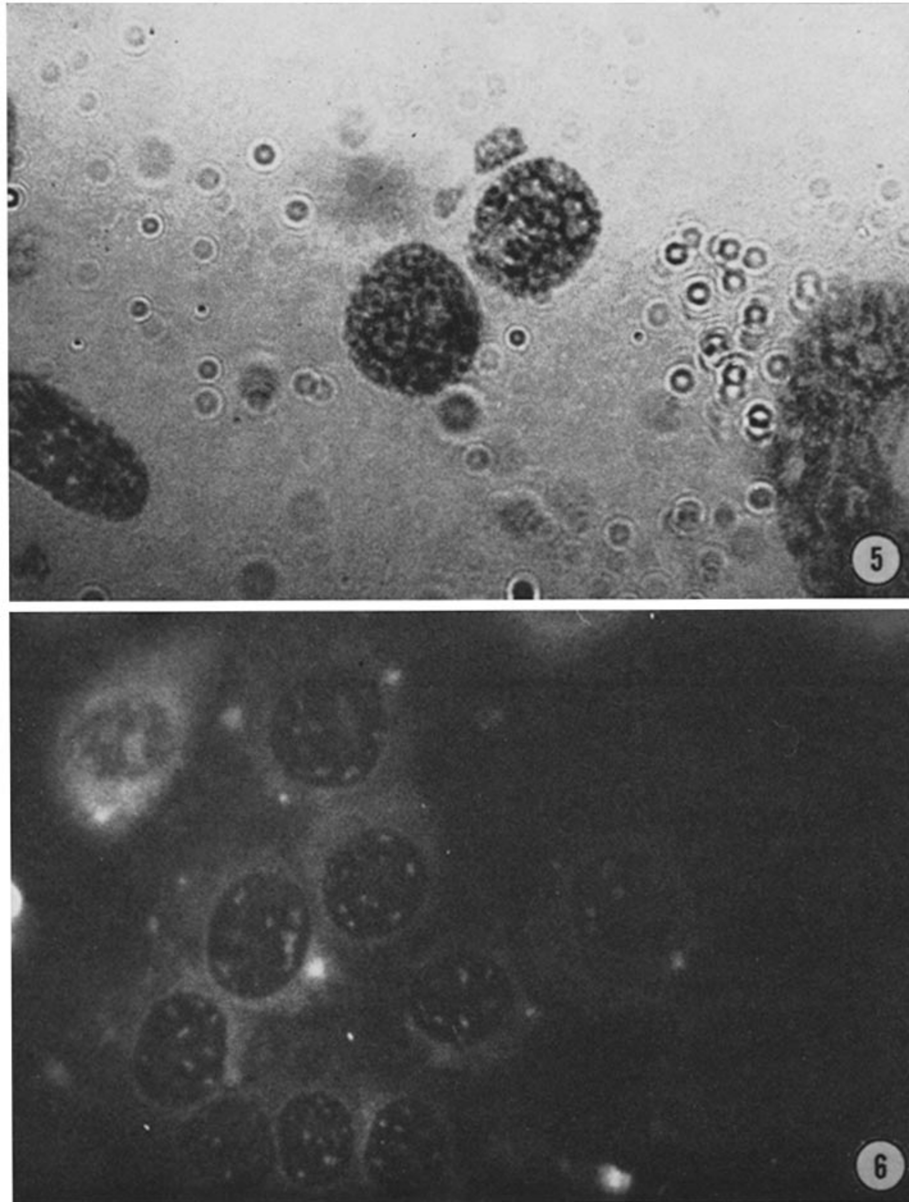

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Plate 4

Fig. 7. Absence of nuclear fluorescence in DNase-treated cells stained with fluore ceinated anti-U. × 2400.

Fig. 8. Diffuse cytoplasmic staining in anti-BSA–treated cells. × 2400.

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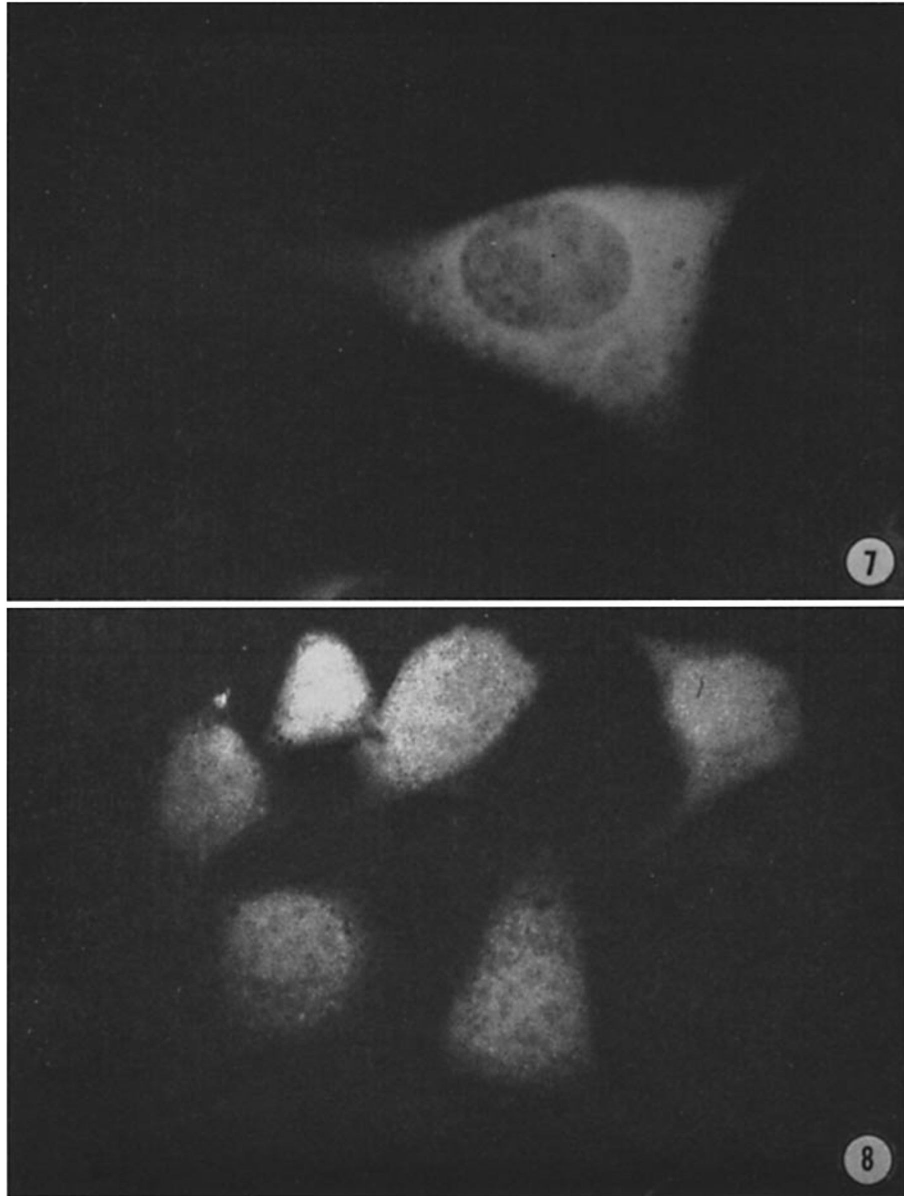

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