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NON-CYTOTOXICITY OF "NUCLEAR ANTIBODIES" FROM LUPUS ERYTHEMATOSUS SERA IN TISSUE CULTURE*

BY

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Systemic lupus erythematosus (SLE) has been characterized as a disorder of unusual immunological responsiveness (Holman, 1959). The multitude of serological abnormalities found in this disorder has suggested an auto-immune mechanism. While some of the abnormal serum factors ("auto-antibodies") may exert a role in producing disease manifestations, such as thrombocytopenia, others, such as the anti-nuclear factors, have not been shown to have pathogenetic properties. One way to examine the concept of a break in immunological self-recognition and subsequent production of harmful tissue-reacting antibodies is to test the degree of damage these anti-nuclear factors produce when they are permitted to react with viable human cells in an isolated system. Lachmann (1961) exposed freshly trypsinized HeLa cells to SLE sera and was unable to detect any effect on multiplication of these cells in tissue culture. Furthermore, he noted a failure of the sera to enter or bind with viable cells. Rapp (1962) demonstrated consistent binding of SLE serum with nuclear components of air-dried (non-viable) tissue culture cells and confirmed Lachmann's observation that SLE anti-nuclear factors did not bind with viable cells. Similarly, Beutner, Sullivan, Barnes, and Witebsky (1961) have shown failure of SLE serum to bind with monkey-kidney cells, or to impair the growth of cells in tissue culture (Beutner and others, 1961).

This report describes our studies in which we have:

(1) Examined the site of cell-binding of SLE globulin in viable and non-viable tissue culture cells;

(2) Evaluated the effect of SLE serum on viable tissue culture cells.

Morphological changes observed in cell monolayers and interference with growth based on the ability of cells to attach and proliferate on glass surfaces were the parameters used to measure cytotoxicity (pathogenicity). To evaluate the adequacy of the methods employed, parallel studies were performed with anti-cell rabbit serum produced by immunizing rabbits with the specific tissue culture cells.

Materials and Methods

Sera.—Blood was collected from patients with clinical SLE and positive L.E.-cell preparations, with sterile procedures used throughout. The serum was promptly separated, inactivated at 56° C. for 30 minutes, and frozen until used. Sera containing specific antibodies against FL (human amnion, continuous passage) cells was prepared in rabbits according to the method of Oda and Puck (1961). Periodic samples of blood were taken *via* ear vein to ascertain the anti-FL cell antibody titre. When adequate titres were reached in a given rabbit, the animal was exsanguinated by cardiac puncture, the sterile sera separated, inactivated at 56° C for 30 minutes, aliquoted, and frozen until used. Complement was processed as described above. Control sera were obtained from normal humans, rabbits, and pooled guinea-pig serum.

Tissue Culture.—FL cells were used exclusively in these studies. Growth media consisted of 83 per cent. Hanks' balanced salt solution with 5 per cent. each of undiluted, inactivated calf and lamb serum, 1 per cent. penicillin and streptomycin solution containing 10,000 units and 0·01 g. respectively, 1 per cent. Amphotericin B (Fungizone, Squibb) solution containing 0·0001 g./ml., and 5 per cent. of 2·8 per cent. $NaHCO_3$ adjusting the pH of the media to 7·4 to 7·6. Standard tissue culture techniques were used for cell handling and trypsinization (Hanks, 1955).

Cells were grown on microscope slides in screw-cap bottles, allowing 2 or 3 days for attachment and growth of a confluent monolayer of cells over the entire surface of each slide.

The media was then replaced with fresh growth media to which was added one of the several sera to be tested. Controls included normal human serum, rabbit serum, and pure growth media. After 2 days, the slides were

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removed and examined both as to site of antibody binding, using the indirect immunofluorescent technique (*vide infra*), and morphologically for evidence of cytotoxicity (microscopic examination after routine haematoxylin and eosin staining). Cytotoxicity was considered present if any, or several, of the following were observed:

(1) Detachment of cells from glass;
(2) Retraction of protoplasmic processes ("rounding");
(3) Alterations, either quantitatively or qualitatively, of cytoplasmic granularity;
(4) Nuclear alterations or loss.

Known numbers of viable freshly-trypsinized FL cells were added to a series of sterile screw-capped tubes containing growth media and one of several sera to be tested, either with or without complement (Table I). After incubation for 72 hours at 36° C., they were examined microscopically and evaluated for attachment to glass and growth (Fig. 1, opposite). An arbitrary grading system was as follows:

1+ Clusters of one to five rounded cells found attached on tube surface, such scant clusters found only on searching several low-power fields;
2+ Five to ten cells (usually rounded) found attached and in small dense groups, representing probable site of original cell attachment and limited subsequent proliferation;
3+ Greater than ten cells, rounded or fibroblastic, found predominantly in fairly dense groups with good evidence of proliferation outward from clone centre, yet lacking sufficient growth to produce a confluent monolayer;
4+ Confluent monolayer growth with predominant fibroblastic cell form (Fig. 1).

Indirect Immunofluorescent Technique.—An indirect immunofluorescent technique was employed to demonstrate the binding sites of SLE and anti-FL rabbit serum (Coons, 1956; Cherry, Goldman, and Carski, 1960). The cells were incubated for 30 minutes with the serum to be tested, followed by triple washings with phosphate buffered saline (pH 7·4). The cells were then incubated again for 30 minutes with anti-human (or anti-rabbit if the primary incubation was with rabbit serum) gamma globulin, prepared in goats and conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratories). Following the second half-hour incubation, the cells were again triple washed as before, and cover slips were mounted in phosphate buffered glycerin (pH 7·6). Positive and negative control sera were uniformly included, allowing detection of non-specific fluorescence of labelled secondary anti-human or anti-rabbit serum. The cells were observed for fluorescence using a Leitz microscope equipped with a dark-field condenser, and Philips HBO 200 mercury lamp as a light source.

Slides with monolayers of FL cells were removed from their growth media, and washed three times both with Hanks' balanced salt solution and isotonic saline. Slides designated as "non-viable cells" were removed from their final saline rinse and air-dried in a 37° C. incubator. After such drying, these non-viable cells were tested against several primary sera (SLE, anti-FL cell, normal human, and rabbit) using the indirect fluorescent technique to locate binding sites. Slides containing viable cells were removed from their final saline rinse and quickly exposed to the same group of primary sera following the same procedure as with the non-viable cells.

Results

Localization of Antibody Binding Sites.—Marked differences were consistently observed in the binding sites of the SLE and anti-FL cell sera. Anti-FL cell rabbit antibody was observed to bind FL cells

TABLE I

Series No.	Description	Tube Contents (ml./tube)											
		AFL		SLE		NHS		NRS		GPC		Mel. GM	
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
1	C′ + Anti-FL	0·12	4·0	0		0		0		0·06	2·0	2·82	94
2	C′ + SLE	0		0·12	4·0	0		0		0·06	2·0	2·82	94
3	C′ + Anti-FL + SLE	0·12	4·0	0·12	4·0	0		0		0·06	2·0	2·70	90
4	C′ + Normal Human	0		0		0·12	4·0	0		0·06	2·0	2·82	94
5	C′ + Normal Rabbit	0		0		0		0·12	4·0	0·06	2·0	2·82	94
6	Anti-FL	0·12	4·0	0		0		0		0		2·88	96
7	SLE	0		0·12	4·0	0		0		0		2·88	96
8	Anti-FL + SLE	0·12	4·0	0·12	4·0	0		0		0		2·76	92
9	Normal Human	0		0		0·12	4·0	0		0		2·88	96
10	Normal Rabbit	0		0		0		0·12	4·0	0		2·88	96
11	C′ Control	0		0		0		0		0·06	2·0	2·94	98
12	Mel. GM Control	0		0		0		0		0		3·00	100

AFL = Anti-FL Sera (undiluted).
SLE = Systemic Lupus Erythematosus (undiluted).
NHS = Normal Human Sera (undiluted).
NRS = Normal Rabbit Sera (undiluted).
GPC = Guinea-Pig Complement diluted 1:10 with Melnick's Growth Medium.
Mel. GM = Melnick's Growth Medium.
C′ = GPC

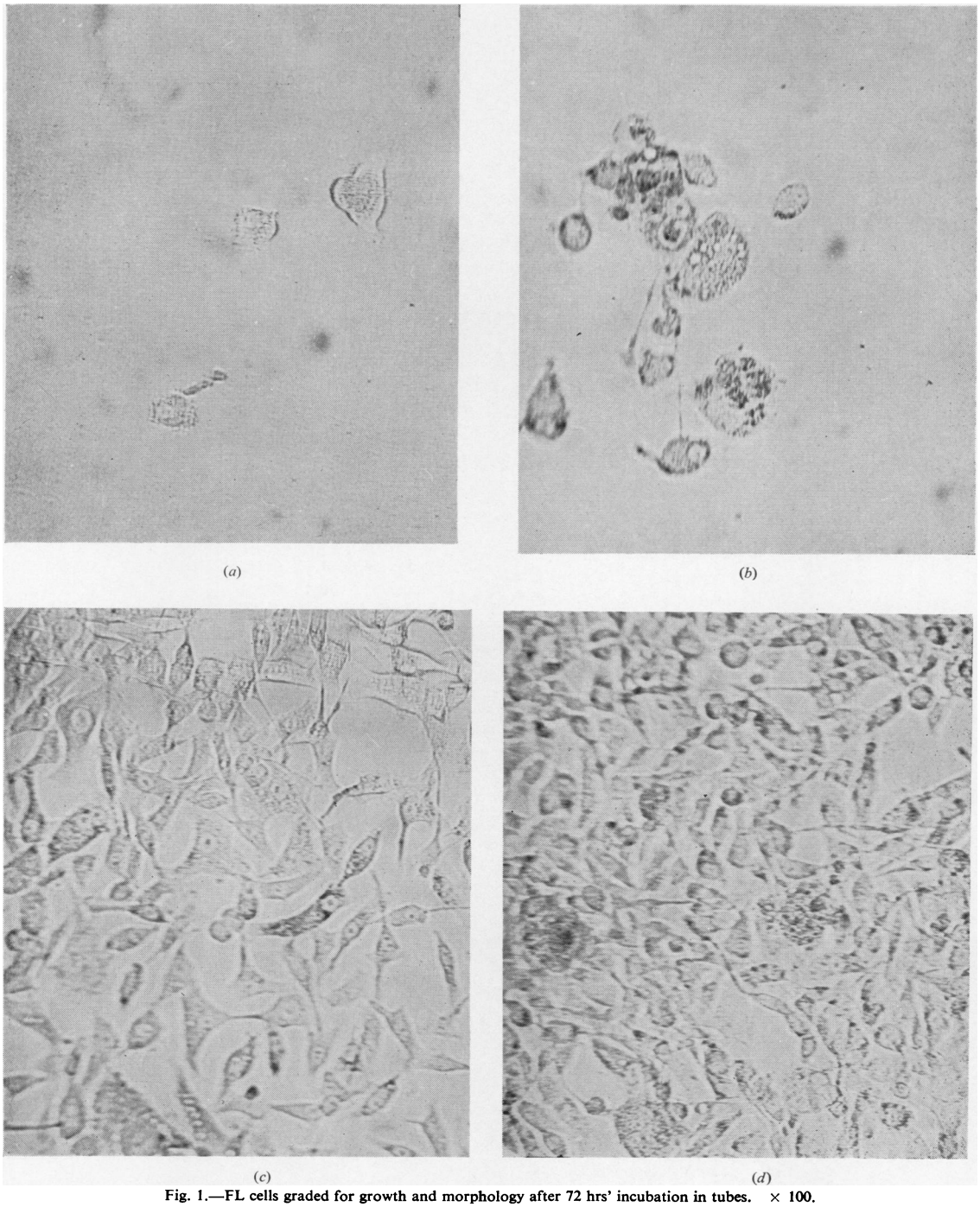


Fig. 1.—FL cells graded for growth and morphology after 72 hrs' incubation in tubes. × 100.
(*a*) 1+ growth—scant clusters of 1 to 5 rounded cells;
(*b*) 2+ growth—small clones of 5 to 10 rounded cells;
(*c*) 3+ growth—large clones with many cells showing fibroblastic proliferation;
(*d*) 4+ growth—confluent cell monolayer with predominance of fibroblasts.

primarily at the cell membrane and variably to the cytoplasm. This reaction occurred with these cells both in the viable and non-viable state (Fig. 2*a* and *b*, opposite).

SLE sera binding was seen only in non-viable cells and involved the nuclear structures, the nuclear membrane, or the entire nucleus and the nucleolus (Fig. 2*c*, opposite). When SLE sera was incubated with viable cells, no nuclear or cytoplasmic binding occurred and the cells appeared exactly as negative controls. In both the negative controls and viable cells incubated with SLE sera, the fluorescence was too faint to be photographed.

Antibody Cytotoxicity to Cells Grown on Slides.—Examination of the haematoxylin and eosin-stained FL cells demonstrated marked differences in the degree of cytotoxicity of the various sera studied. Cells which were grown in media containing SLE, normal human, and normal rabbit serum, or Melnick's growth medium, showed little or no evidence of cytotoxicity, *i.e.* no detachment from the slide surface, limited amount of "rounding" involving only a few cells, with some delicate cytoplasmic granularity (Fig. 3*a*, overleaf). In contrast, cells exposed to sera containing anti-FL cell antibody were markedly altered as indicated by universal "rounding", coarse cytoplasmic granulation, many atypical "giant" cells, and cells lacking nuclei (Fig. 3*b*, overleaf).

Antibody Cytotoxicity to Cell Attachment and Subsequent Growth in Tubes.—The results of this study are shown in Table II. Anti-FL cell rabbit serum is the only serum evaluated which was found to influence the attachment and subsequent growth of these cells. In addition, it is noteworthy that this reaction is enhanced by the presence of complement (comparing results of Series 1 and 6, Table I). Complement alone appears to have some inhibitory influence in and of itself. Most significant is the consistent observation that SLE sera, with anti-nuclear factor, has no apparent effect on the attachment and further proliferation of these cells as tested.

TABLE II

Series Number	Description of Tube Contents	Growth Evaluation
1	C′ + Anti-FL Cell Rabbit Serum	1+
2	C′ + SLE Serum	4+
3	C′ + Anti-FL Cell Rabbit Serum + SLE Serum	2+
4	C′ + Normal Human Serum	4+
5	C′ + Normal Rabbit Serum	4+
6	Anti-FL Cell Rabbit Serum	3+
7	SLE Serum	4+
8	Anti-FL Cell Rabbit Serum + SLE Serum	3+
9	Normal Human Serum	4+
10	Normal Rabbit Serum	4+
11	C′ Control	3+
12	Melnick's Growth Medium Control	4+

C′ = Complement.
SLE = Systemic Lupus Erythematosus.

Discussion

Numerous humoural abnormalities have been described in patients with SLE (Holman and Kunkel, 1959; Doniach and Roitt, 1962; Shulman, 1963). Antibody-like globulins which are capable of reacting with saline extracts of human liver (Mackay and Gajdusek, 1958; Asherson, 1959), thyroid (Hijmans, Doniach, Roitt, and Holborow, 1961), and cell nuclei or nuclear constituents (Shulman, 1963) have contributed to the concept that this disease is autoimmune in nature. However, there is growing evidence that anti-nuclear factor plays no primary pathogenetic role. The transplacental transfer of L.E.-cell factor has been reported without producing disease (Bridge and Foley, 1954). Patients with SLE may have positive tests for anti-nuclear factors yet exhibit mild disease. Finally, antibodies to DNA have been experimentally produced in animals without precipitation of disease (Miescher, Cooper, and Benacerraf, 1960).

Beutner and others (1961) searched for binding of gamma globulin in oral mucosa cells and liver biopsy tissue in patients with nuclear antibodies. Using the direct and indirect fluorescent antibody methods, they could not demonstrate *in vivo* the nuclear localization of gamma globulin. Lachmann (1961), Rapp (1962), and Buetner and others (1961) have reported the inability of SLE anti-nuclear factors to bind with nuclei of viable cells. Furthermore, SLE sera had no observable effect on the multiplication of cells grown in tissue culture. Our studies have confirmed these observations, namely that SLE serum which is reactive with cell nuclei of non-viable cells does not react with nuclei of viable cells in tissue culture. SLE serum does not interfere with attachment of cells to glass or growth of cells, and does not produce morphologic changes. In contrast, specific anti-cell serum produced in rabbits not only binds with cell membrane of viable cells, but is cytotoxic.

Available data therefore suggests that, while anti-nuclear serum factors are characteristic of SLE, these "antibodies" have not been shown to react with living cells nor produce injury. It seems reasonable to suppose that these factors cannot be implicated in the direct pathogenesis of SLE but should rather be considered as consequential to a more fundamental and poorly defined disturbance of immunological hyper-reactivity.

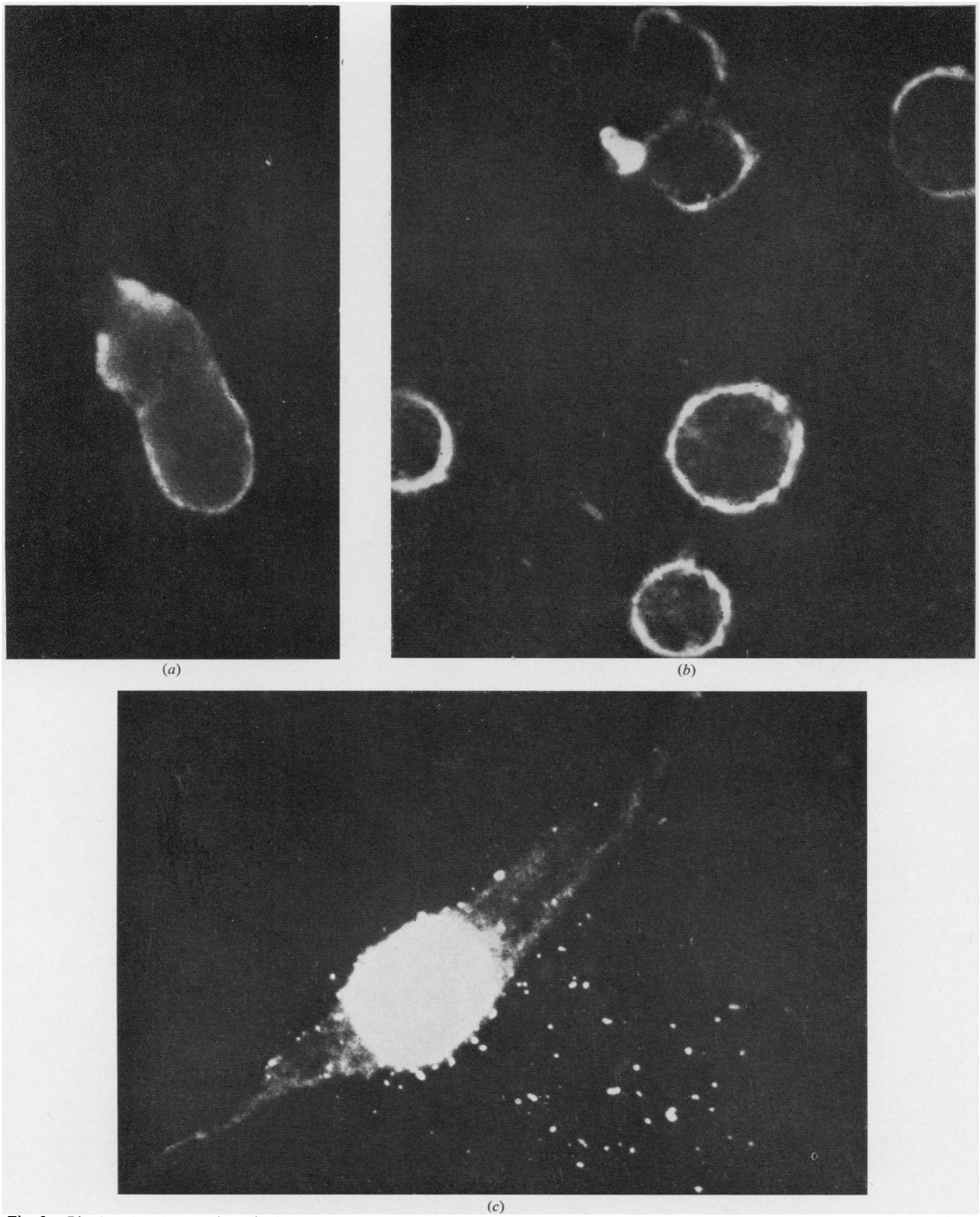
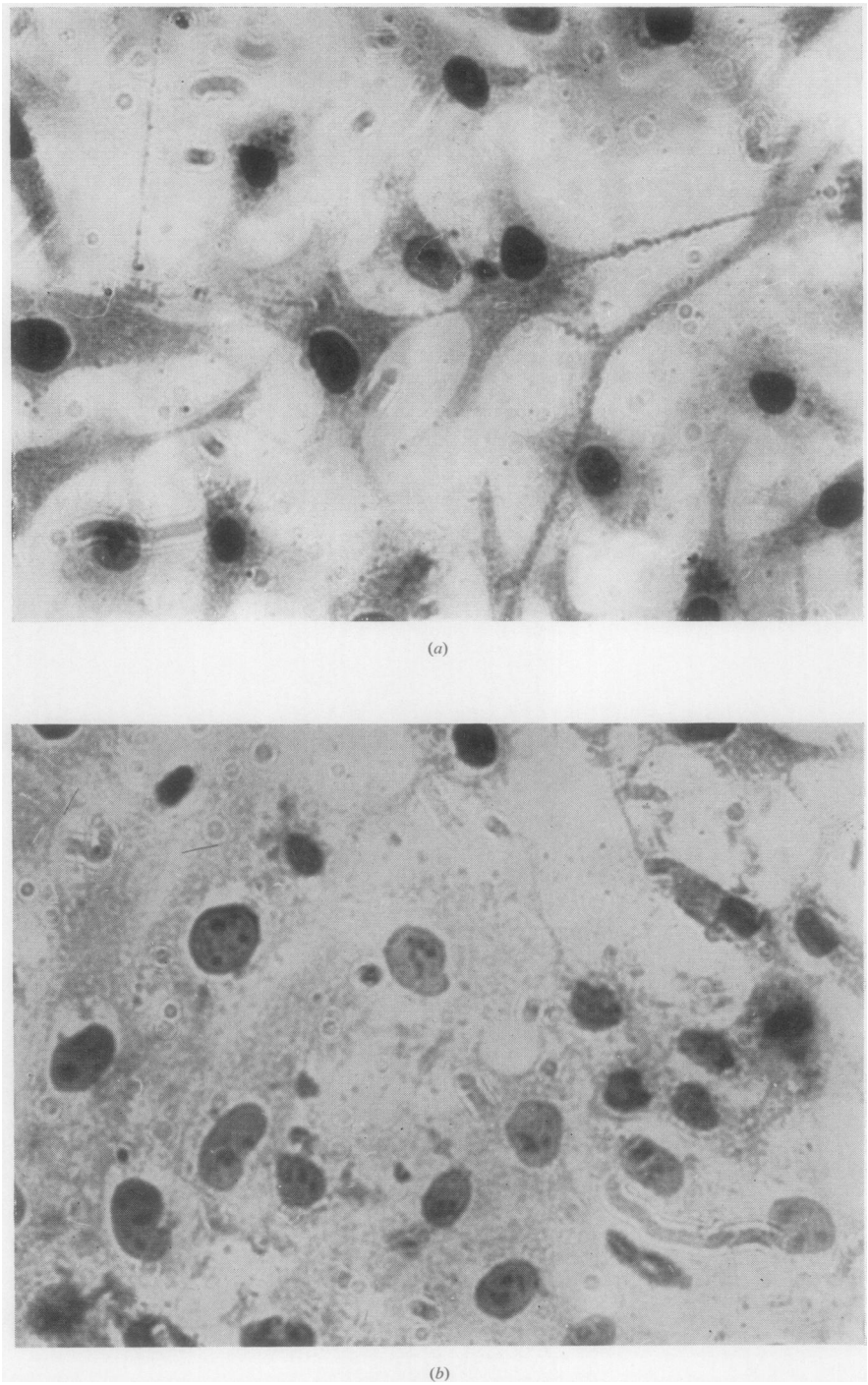


Fig. 2.—Binding sites for rabbit anti-FL serum and SLE serum to FL cells are demonstrated by the indirect immunofluorescent technique. × 1,000.

(*a*) and (*b*) Rabbit anti-FL serum is localized at the cell membrane in viable cells and non-viable (air-dried) cells;
(*c*) SLE serum binds with nucleus but only in non-viable cells.



(*a*)

(*b*)

Fig. 3.—Effect of SLE serum and rabbit anti-FL serum on established cell monolayers illustrated. × 450.
(*a*) SLE serum produced no discernible morphologic change.
(*b*) Rabbit anti-serum induced rounding of cells. Haematoxylin and eosin.

Summary

The localization of binding sites of antibodies in sera from SLE patients was determined by indirect immunofluorescent techniques and compared with the locus of binding of specially-prepared FL cell antisera on both living and dead tissue culture cells. Anti-FL cell antibodies uniformly localized peripherally, involving only the cell membrane and cytoplasm of both viable and non-viable cells. SLE sera were found to bind with the nucleus of non-viable cells only.

The failure of the SLE serum to react with viable cells was further studied by morphological examination of cells stained with haematoxylin and eosin which had been previously exposed to SLE and FL cell rabbit antisera. Viable tissue culture cells exposed to SLE antibodies were identical with normal controls, indicating that there was no cytotoxicity associated with the SLE sera.

Finally, SLE serum cytotoxicity was evaluated by incubating freshly trypsinized FL cells in tubes with a series of different sera, with and without complement. After 72 hours, these cells were evaluated as to their degree of attachment and subsequent growth on the tube surface. Again, SLE antibodies were found to be non-toxic to these tissue culture cells. In contrast, anti-FL cell antibodies displayed marked cytotoxicity both on the slides and in tubes, especially in the presence of complement.

These results demonstrate that anti-nuclear factors in SLE serum do not damage human amnion cells in tissue cultures. The inference is drawn that these factors cannot be implicated in the direct pathogenesis of SLE.

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Non-cytotoxicité des "anticorps antinucléaires" des sérums de lupus érythémateux en culture tissulaire

RÉSUMÉ

On détermina par des procédés d'immunofluorescence indirecte les lieux d'agrégation des anticorps dans les sérums des malades atteints de lupus érythémateux disséminé (LED = *SLE*) et on les compara aux lieux d'agrégation des antisérums spécialement préparés contre les cellules FL (amniotiques humaines) dans des tissus de culture vivants et morts. Les anticorps anti-FL s'agrégeaient uniformément à la périphérie, engageant seulement la membrane et le cytoplasme des cellules tant viables que non-viables. Les sérums LED n'engageaient que le noyau des cellules non-viables.

L'absence de la réaction entre le sérum lupique et les cellules viables fut aussi étudiée par l'examen morphologique des cellules colorées par l'hématoxyline et l'éosine, après les avoir exposé aux antisérums LED et anti-FL de lapin. Les cellules de culture viables exposées aux anticorps LED ne se distinguaient pas de cellules normales, indiquant ainsi qu'il n'y avait pas de cytotoxicité associée aux sérums LED.

Finalement, la cytotoxicité du sérum LED fut évalué par l'incubation des cellules FL fraichement tryptolysées avec une série de différents sérums, avec ou sans complément. Après 72 heures, ces cellules furent évaluées en ce qui concerne leur agrégation et leur croissance à la surface du tube. Une fois de plus on trouva que les anticorps LED n'étaient pas toxiques pour ces cellules de culture. D'autre part, les anticorps anti-FL manifestaient une nette cytotoxicité tant sur les lames que dans les tubes, surtout en présence du complément.

Ces résultats démontrent que les facteurs antinucléaires dans le sérum de LED n'endommagent pas les cellules de culture de l'amnios humain. On en conclut que ces facteurs ne peuvent pas être impliqués directément dans la pathogénie du LED.

Ausencia de citotoxicidad de los "anticuerpos antinucleares" de los sueros de lupus eritematoso en tejidos de cultivo

SUMARIO

Se determinaron por métodos de immunofluorescencia indirecta los lugares de agregación de los anticuerpos en los sueros de enfermos con lupus eritematoso diseminado (LED = *SLE*) y se compararon estos lugares con los de agregación de antisueros especialmente preparados contra células FL (amnióticas humanas) en tejidos de cultivo vivos y meurtos. Los anticuerpos anti-FL se agregaban uniformemente a la periferia, implicando solamente la membrana y el citoplasma de las células

tanto viables, como non-viables. Los sueros LED implicaban sólo el nucleo de las células non-viables.

La ausencia de reacción entre el suero LED y las células viables fué tambien estudiada por el examen morfológico de células coloradas con hematoxilina y eosina, después de haberlas expuesto a antisueros LED y anti-FL de conejo. Las células de cultura viables expuestas a los anticuerpos LED no se distinguieron de las células normales, indicando de este modo que no hubo citotoxicidad asociada con el suero LED.

Finalmente, la citotoxicidad del suero LED fué investigada por incubación de células FL recientemente triptolizadas con una serie de diferentes sueros, con o sin complemento. Después de 72 horas estas células fueron apreciadas respecto a su agregación y su crecimiento a la superficie del tubo. Una vez más se vió que los anticuerpos LED no fueron tóxicos para las células de cultura. En cambio, los anticuerpos anti-FL manifestaron una citotoxicidad pronunciada tanto sobre los portaobjetos como en los tubos, en particular en presencia de complemento.

Estos resultados muestran que los factores antinucleares en el suero de LED no dañan las células de cultura del amnios humano. Se concluye que estos factores no se pueden implicar directamente en la patogénesis del LED.