

ON THE NATURE OF THE "LIPOVIRUS"*

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During an attempt to isolate viral agents from the bloods of infectious hepatitis patients, an unusual transmissible agent was encountered (1). Since the activity of this agent was apparently protected by a lipid-rich outer membrane, and since a lipogenic toxin was consistently found in inoculated human cell culture, the name "lipovirus" was proposed tentatively (2, 3). This name was subsequently used with quotations to signify the uncertainty of and the necessity for further investigation on the nature of this agent.

Experimental data which lead to the formulation of the following concept have now been obtained. The "lipovirus" is a transferable factor(s) which is carried by an ameboid cell. The ameboid cell is capable of transferring the factor(s) to human cells by an unknown mechanism which requires cell-to-cell contact. Some human cells which have accepted the factor(s) develop characteristic nuclear changes, accumulate specific antigens, and eventually disintegrate. The exact nature of this transferable factor(s) remains to be investigated. There is no direct evidence that the recipient human cell can transfer the factor(s) to other human cells, or that the factor(s) can alter a human cell into an ameboid cell. Data in support of this concept are presented in this and the accompanying manuscript (4).

To avoid introducing a new name for a transferable factor(s) whose exact nature remains to be elucidated, we would like to suggest that the term "lipovirus" (used with quotations) be temporarily retained for this transferable factor(s), and that the ameboid cell carrying this factor(s) be temporarily referred to as the ameboid cell-"lipovirus" complex, or, simply, AL complex. We are aware of the fact that the characterization of the "lipovirus" is still incomplete and that the criteria proposed currently for a virus (5) have not yet been fulfilled. However, to introduce a new and temporary name for this factor at this time would certainly cause further confusion in this area of in-

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vestigation. It should be emphasized that the main objectives of this manuscript are to clarify some of the confusion relating to this complex phenomenon and to interest other investigators in examining this phenomenon critically.

Materials and Methods

Cell Cultures.—The cells were from a line derived from human hepatic tissue (6), from 11-day-old chick embryos, and from the human amniotic membrane. These cells are referred to hereafter as Lich, CE, and HA, respectively. Cell suspensions were prepared by the standard trypsinization procedure, and the cells were grown on glass as “monolayers” in test tubes (18 × 150 mm), containing 1 ml of standard medium. The cultures were placed on a roller (12 revolutions per hour) at 36°C.

Nutrient Media.—Two culture media were employed. A *standard medium* consisted of 5% inactivated calf serum in Eagle's basal medium (7) as modified (8). An *enriched medium* consisted of 10% inactivated calf serum in modified Eagle's basal medium with 0.5% yeast extract and 0.5% casein hydrolysate (Trypticase, BBL). The antibiotic mixture used in both media consisted of staphicillin, streptomycin, polymixin B, and mycostatin at concentrations of 50, 50, 5, and 25 µg/ml of medium, respectively.

Ameboid Cell-“Lipovirus” Complex (AL Complex).—The AL complex was encountered in 1954 when the newly established Lich cells were inoculated with fresh hepatitis bloods (1). Subsequent attempts to reisolate a similar agent have been unsuccessful. The AL complex was propagated serially and exclusively in the Lich culture by transferring fluid from a previously inoculated culture which had shown characteristic cytopathic change. No attempt was made to remove whole cell or cell debris from the fluid used for making passages. This passage material was to be referred to as stock inoculum. It has undergone innumerable passages since 1954.

To eliminate the possibility that some extraneous agents may have been introduced inadvertently into the stock inoculum during these innumerable passages, we “purified” the stock inoculum by terminal dilution as described in the titration of tissue culture units. At the termination of a titration, the Lich culture that had received the least amount of inoculum and yet showed characteristic cytopathic changes was used for a second titration. This procedure was repeated serially five times. This fifth serial passage material was then used as inoculum for a large number of Lich cultures. After the appearance of complete cytopathic changes, the fluid and cells were pooled and kept at 0°–4°C. This pool of material is to be referred to as purified inoculum.

It has been shown that the ameboid cells are capable of further multiplication in the standard tissue culture medium without tissue cells if the medium is enriched with yeast extract and Trypticase (9). A line of AL complex which has been passed serially in the enriched medium for over three years was also used in many experiments, such as the determination of the presence of DNA in the cytoplasm of these cells by radioautography and in immunologic study.

Hartmannellid Amebae.—For comparative study, a strain of Hartmannellid ameba (Lilly strain) was obtained from Dr. T. Dunnebacke of the Virus Laboratory, Berkeley, California. The strain was said to have originated in a monkey kidney tissue culture (10) and was kept in Dr. W. Balamuth's laboratory for many years in a medium containing the following compounds: 20 g proteose-peptone, 2 g yeast extract, 1.4 g sodium citrate, 19 mg FeNH₄(SO₄)₂, 100 mmoles glucose, 4 mmoles MgSO₄, 0.4 mmoles CaCl₂, 1 mmole Na₂PO₄, 4 mmoles KH₂PO₄, and 1000 ml of H₂O. After being received in this laboratory, the amebae were transferred to the enriched medium.

Testing for PPLO and Other Microbial Contaminants.—Tissue cultures, the AL complex, and the Hartmannellid amebae were tested at intervals for PPLO by the method of Chanock,

Hayflick, and Barle (11), for bacteria on nutrient blood agar and in thioglycolate broth, and for fungi on Sabouraud's medium. No contamination by these organisms was encountered.

Quantitation of the AL Complex.—Cell number was determined with a hemocytometer. Cells were first dispersed by pipetting, two or four different counts were made, and the means of the counts were calculated. Based on the results for four cultures, the standard deviations were found to be about 10 to 20% of the means. *Tissue culture units* were determined by the standard terminal dilution method and calculated according to the method of Schwerdt and Merrell (12). Suspensions of ameboid cells were diluted at an increment of 0.5 log, and four tissue cultures were inoculated with 0.1 ml from each dilution. Inoculated cultures were kept at 32°–35°C and were examined for cytopathic changes for at least 14 days. The standard errors for four consecutive titrations, calculated according to Pizzi's formula (13), were 0.25 to 0.3 log.

Radioautogram.—Cells grown on cover slips, after appropriate treatment, were dipped in a photographic emulsion (Ilford G5, diluted 1 to 2 with H₂O). After 5 days of exposure at 0°–4°C, the cover slips were developed with Kodak dectol developer, fixed with Kodak acid fixer, and then stained with hematoxylin and eosin. Enzymes used in the radioautographic experiments were deoxyribonuclease (twice crystallized) and ribonuclease (5 times crystallized). Both were purchased from the Nutritional Biochemical Corporation, and were used at a concentration of about 0.05 to 0.1 mg/ml in Eagle's basal medium with pH adjusted to 6–6.5.

Serological Procedure.—The complement-fixation (CF) test was that routinely used in most laboratories (14). The reacting mixture consisted of 0.1 ml of serum, 2 units of antigen in 0.1 ml, and 1.5 to 2 exact units of complement in 0.2 ml. The mixture was kept at 0°–2°C for 18 to 20 hr. 0.2 ml of 1% sensitized erythrocytes was added. After 37°C for 30 min, the degree of hemolysis was estimated by comparing with a set of standards. Tubes showing 0 to 50% hemolysis were arbitrarily considered positive. The antibody titer was expressed as the reciprocal of the highest serum dilution which gave a positive reaction. Pooled guinea pig serum, kept frozen at below –60°C, was used as complement.

The sensitized erythrocyte agglutination (SEA) test was similar to that described previously (15) except for one modification. The sensitizing antigen was concentrated fivefold by precipitation with 2 volumes of ethanol at 0°–2°C for 18 to 20 hr. With this modification, satisfactory sensitizing antigens were obtained consistently from Lich cultures inoculated with the AL complex by the procedure described previously (15). The sensitivity of the SEA test was also enhanced by the use of the concentrated antigen.

Antiserums against the AL complex or against the Hartmannellid amebae were prepared by intravenous inoculation of rabbits at weekly intervals. The AL complex or the Hartmannellid amebae were grown in the enriched medium and were washed three times in 0.85% NaCl. About 3×10^6 cells suspended in 0.85% NaCl were given to each animal at each inoculation. The animals were bled 5 days after the fourth inoculation. Similar cell suspensions were used for the preparation of CF antigens. The cell suspensions were frozen and thawed three times and then heated at 56°C for 10 min. They were then standardized with appropriate antiserums by the "checker board" titration method (14). The highest dilution of a cell suspension which gave the maximum titer for an antiserum was considered as 1 unit. 2 units of antigen were used in tests with pre- and postimmunization serum. In tests with human sera (16), 8 to 16 units of antigen were used.

RESULTS

Morphologic Changes in Lich Cultures Inoculated with the AL Complex.—5 days after the inoculation of three tissue culture units of the AL complex, several small foci of round granular cells were noted (see Fig. 1 a). 1 to 2 days

later, about half of the cell sheet was involved with rounded and ameboid cells (Fig. 1 *b*). In 1 more day, the Lich cells were completely replaced by the ameboid cells (Fig. 1 *c*).

Examination of fixed and stained preparations of inoculated Lich culture, with about 10% involvement of the cell sheet, revealed several types of nuclear abnormalities. The most frequently observed changes were "clumping" and "margination" of basophilic materials (Figs. 2 *a* and 2 *b*). Less frequently observed were the peculiar arrangement of basophilic material around the nucleolus (Fig. 2 *c*), the shrunken nucleus (Fig. 2 *d*), and the basophilic ring around the nucleolus (Fig. 2 *e*). Cells with changes shown in Figs. 2 *a*, 2 *c*, and 2 *d* but with no visible nuclear membrane were also seen. All nuclei in a multinucleated cell, if involved, showed similar morphologic abnormality. The nucleus of the ameboid cell appeared as a small basophilic ring surrounding a basophilic dot (Figs. 2 *f* and 2 *g*) which resembled the nucleus of an ameba (Fig. 2 *h*). The nuclear changes involved primarily Feulgen-positive materials as shown in Figs. 3 *a* to 3 *d*. Of special interest was the Feulgen reaction of the ameboid cell; only the basophilic ring was Feulgen-positive (Fig. 3 *d*). This distribution of Feulgen-positive material differed from that described for several species of amebae associated with man (17).

To determine the interval between inoculation and the appearance of nuclear lesions, cultures containing about 200,000 Lich cells were inoculated with about 100,000 ameboid cells. After 2 hr, the inoculated cultures were washed twice with the standard medium to remove some of the ameboid cells, fed with fresh medium, and incubated at 35°C. 2, 4, 8, and 12 hr after inoculation, cultures were fixed, stained, and examined for nuclear lesions. The number of Lich cells with and without nuclear abnormality was scored for 20 random fields with a 40 × objective.

The percentages of Lich cells with characteristic nuclear lesions (Figs. 2 *a*, 2 *b*, 2 *c*, and 2 *e*) were found to be 0, 1.5, 16.3, and 21.5, respectively, for the 2, 4, 8, and 12 hr cultures.

Sequential Changes as Seen in the Time-Lapse Cinematography.—Cells in inoculated Lich culture were seen first to undergo nuclear shrinkage and then cytoplasmic retraction thereby appearing as round cells with small nuclei (Fig. 4 *a* to 4 *f*). Vacuolation of cytoplasm and nucleoli often, but not invariably, preceded the shrinkage of nuclei. Ameboid cells were seen wandering in and out of the field, making contact with the Lich cells. The nuclear shrinkage and cell-rounding may occur without further contact with ameboid cells. The round cells were no longer adherent to glass and were seen to drift in and out of the field, making further sequential observation difficult. In preparations in which many ameboid cells were present in the field of observation, rupturing of rounded cells was seen. An occasional rounded cell of unknown origin was also seen to assume the shape and movement of the ameboid cells. Phagocytosis of cell debris by the ameboid but not by the Lich cells was observed. The ame-

boid cells were seen to divide by binary fission. By scoring the average number of ameboid cells and the number of binary fissions in the field of observation for any 24 hr sequence, the average frequency of division was estimated to be once in 1 to 4 days.

In a single successful study of a sparsely populated Lich culture inoculated with a few ameboid cells, an ameboid cell was seen to crawl over and around a Lich cell for about 35 min and then move out of the field. About 29 hr later, the Lich cell divided. One of the daughter cells became rounded 7 hr after the division. In another 6.5 hr, the other daughter cell also became rounded. Both remained as rounded cells for the remainder of the experiment. Other Lich cells in the vicinity, which had not been in contact with the ameboid cells, continued to divide and retain their polygonal shape for another 35 hr when the experiment was terminated (Figs. 6 a to 6 j).

Failure to Induce Cytopathic Changes in Lich Cultures Inoculated with Cell-Free Inoculi.—To determine if nuclear changes within the Lich cells may be caused by toxic products of the ameboid cells, we tested filtrates of cultures of AL complex in enriched medium or in tissue cultures for activity. Ultrafine glass, Sela 02 and membrane (Millipore, 0.45 and 5 μ in pore size) filters were used in these experiments. The test materials were generally filtered without prior treatment. In several experiments, the cells were ruptured prior to filtration by three cycles of freeze-thawing or by exposure to sonic waves for 1 to 5 min. All filtrates were incapable of inducing progressive cytopathic changes or nuclear lesions in Lich, HA, and CE cultures.

The possibility that the ameboid cells may release a labile agent capable of inducing nuclear changes was explored with the following experiment. A lucite ring (2 cm in diameter and 0.8 cm in height) was cemented to a membrane filter (Millipore, 150 μ in thickness and 0.45 μ in pore size) to form a "well." Lich cells were first grown on the external surface of the filter. About 10^8 Lich and 10^8 ameboid cells, each in 0.1 ml of standard media were transferred into the "well" which was then placed on a thin layer of gauze in a Petri dish. Standard medium was added to a depth of 0.5 cm, and the Petri dish was placed in a jar gassed with 5% CO₂ in air. After 3 and 7 days, the filter was fixed with Bouin's solution and stained with hematoxylin. Cells on the external surface were examined microscopically for ameboid cells and for Lich cells with characteristic nuclear lesions. None were found in four consecutive experiments.

The Relationship Between the Number of Ameboid Cells and Tissue Culture Units in an Inoculum.—22 Lich cultures which had been inoculated with the stock or purified inocula and which had shown complete replacement by the ameboid cells were studied. In all cultures, the values for cell number and tissue culture units were approximately similar.

Failure of Binary Divisions of the Ameboid Cells to Account for Increases in Tissue Culture Units of Lich Cultures Inoculated with the AL Complex.—Based

on time-lapse cinematographs that recorded the interaction of the ameboid and Lich cells, the average frequency of division of the ameboid cell was found to be relatively low (one division in every 24 to 96 hr). Yet, it has been possible to demonstrate an increase of about 10^5 tissue culture units of AL complex within 7 days after the inoculation of a CE or Lich culture with three units of AL complex. To explore the basis for this discrepancy, we performed the following experiment. A culture containing about 2×10^6 Lich cells in 5 ml of standard medium was inoculated with 5×10^4 units of AL complex. The culture was monitored with time-lapse cinematography (magnification of 100, phase contrast, 20 sec per frame, temperature 35°C). After 48 hr, the number of tissue culture units of AL complex was determined. The average frequency of division of the ameboid cells was determined by reviewing the films. The movie screen was divided into four quadrants, and four investigators were assigned to score in each quadrant the number of ameboid cells and the number of division. The average number of ameboid cells in the field of observation was determined by scoring these cells once every 50 to 100 frames. The actual number scored for each of the consecutive 50th to 100th frames was 3, 1, 4, 3, 4, 1, 0, 0, 1, 0, 0, 1, 1, 1, 1, 3, 3, 1, 3, 1, 1, 4, 3, 5, 5, 4, 5, 0, 1, 0, 4, 3, 3, 3, 1, 5, 5, 5, 3, 8, 11, 11, 12, 9, 12, 12, 12, 11, 12, 11, 8, 7, 7, 13, 11, 9, 8, 11, 11, 5, 7, 7, 5, 5, 9, 9, 7, 4, 5, 8, 7, 5, 4, 4, 2, 3, 3, 4, 3, 5, 5, 4, 3, 7, 6, 6, 5, 6, 6, 7, 7, 9, 7, 6, 3, 10, 6, 6, 8, 9, 10, 9, 7, 10, 7, 7, 8, 13, 12, 18, 15, 16, 12, 20, 19, and 15. The average number was 6.2 ameboid cells per field. The actual observation time for this sequence of movie was 36.5 hr. Thus, 6.2 ameboid cells have been observed for 36.5 hr. The number of binary division was easily and accurately scored by running the films back and forth several times. Five divisions were observed. Based on these data, the average frequency of division was calculated to be once in every 45 hr. In contrast, the actual increase in tissue culture units for this culture was 1.5 log. The experiment was repeated with similar results; average frequency of division was found to be once in every 33 hr while the increase in tissue culture units was 2 log in 48 hr.

Presence of DNA in the Cytoplasm of the Ameboid Cells.—To explore the possibility that the cytoplasm of the ameboid cell may contain DNA material, we examined the AL complex, which had been grown in the enriched medium without tissue cells for over 3 years, by the following experiment. The ameboid cells were grown on cover slips and were fed with the standard medium which contained $10 \mu\text{c}$ per ml of thymidine- H^3 (specific activity, 6.7 c/mmole). The serum component of the medium was also increased from 5 to 10%. After 24 hr, the cover slips were rinsed three times in Eagle's basal medium, fixed for 30 min with 25% acetic acid in ethanol, rinsed twice with 50% ethanol, and placed in 100 ml of 0.85% NaCl for 2 hr. Each cover slip was then broken into three pieces; one was placed in 2 ml of deoxyribonuclease solution, one in 2 ml of ribonuclease solution, and one in diluent. After 4 hr at 37°C , the cover slips

were washed twice in diluent and thrice in H₂O. The cover slips then were examined by radioautographic technic. As shown in Fig. 5 *a*, there was a concentration of grains over the cytoplasm of the ameboid cells. The number of grains per cell varied considerably. In preparations which were digested with DNase, the number of grains per cell was not significantly higher than background (Fig. 5 *b*). Predigestion with RNase failed to reduce the number of grains per cell. The presence in the cytoplasm of the ameboid cells of thymidine-containing particles that were digestible with DNase but not with RNase was consistently observed in five experiments. Cultures of Lich cells grown in medium containing 1 to 10 μ c thymidine-H³ per milliliter for 24 hr showed high concentration of grains over the nuclei and no apparent concentration over the cytoplasm. Digestion with DNase, but not with RNase, greatly reduced the number of grains over the nuclei of the labeled Lich cell. Based on our present knowledge of the chemistry of nucleic acids, we conclude that there is DNA in the cytoplasm of the ameboid cell. An incidental finding of some interest is the absence of concentration of grains over the nuclei of the ameboid cells in all the radioautographic preparations examined thus far.

Effect of 5-Fluorodeoxyuridine (FUDR) on the Development of Nuclear Lesions.—If the transferrable factor (“lipovirus”) is DNA material and if the characteristic nuclear lesions in the Lich cells is dependent on the replication of this factor within the Lich cells, it should be possible to suppress partially the formation of nuclear lesions by FUDR. This possibility was explored with the following experiment.

About 200,000 Lich cells in 1 ml of standard medium were inoculated into six Leighton tubes each containing a cover slip. After 20 hr at 37°C, the cultures were divided into three sets of two; the media in each of the three sets were replaced with a modified standard medium (the calf serum component was replaced by dialyzed horse serum), the modified standard medium containing FUDR at 10 μ g/ml, and the modified standard medium containing FUDR and thymidine at 10 and 100 μ g/ml, respectively. After 1 hr, 0.2 ml of a suspension of the AL complex was added to each of the cultures. The AL complex was harvested from an inoculated Lich culture which had shown complete cytopathic changes and was washed twice and resuspended in Eagle's basal medium. After 6 hr at 35°C, the cultures were fixed with Bouin's fluid, stained with hematoxylin, given code numbers, and examined for nuclear changes. The number of cells with or without nuclear lesions was scored for each microscopic field with a 40 \times objective, and at least 40 fields were scored for each culture.

Results are shown in Table I. In all four experiments, the percentage of Lich cells with nuclear lesions was lower in cultures containing FUDR than in control cultures or in cultures containing FUDR and thymidine. This reduction of the percentage of Lich cells with nuclear lesion by FUDR as shown in Table I is statistically significant (*P* value is less than 0.001 by the Chi square test).

These results suggest that partial suppression of DNA synthesis also suppresses partially the development of specific nuclear lesions in the inoculated Lich cell.

Immunologic Relation Between the AL Complex and a Hartmannellid Ameba (Lilly Strain).—The resemblance of the nucleus of the ameboid cell to the nucleus of the human ameba or the free-living Hartmannellid ameba has been referred to already. One obvious and important question is whether the AL

TABLE I
5-Fluorodeoxyuridine on the Development of Nuclear Lesions in Lich Cultures Inoculated with AL Complex

Experiment	Media*	No. of Lich cells examined	No. showing nuclear lesion	Per cent showing nuclear lesion
1	Control A	565	102	18.1
	Control B	521	101	19.4
	FUDR A	727	79	10.9
	FUDR B	990	53	5.3
	F + T A	447	91	20.4
	F + T B	564	106	18.8
2	Control A	1000	35	3.5
	Control B	1000	36	3.6
	FUDR A	1000	18	1.8
	FUDR B	1000	16	1.6
	F + T A	1000	36	3.6
	F + T B	1000	45	4.5
3	Control	4000	170	4.3
	FUDR	2000	45	2.3
	F + T	2000	88	4.4
4	Control	3000	241	8.0
	FUDR	3000	158	5.3
	F + T	2000	174	8.7

* F + T = FUDR + thymidine.

complex is one of the known species of amebae. First to be considered are those amebae that are known to be associated with man, especially the pathogen, *Entamoeba histolytica*. These human amebae are known to be difficult to cultivate; they require anaerobic conditions for growth; they need other microorganisms to serve as food; and they form multinucleated cysts (18). Serologic surveys also showed relatively low prevalence of antibody against *E. histolytica* even in those individuals who harbored the ameba in their intestinal tracts (19–21). Since these characteristics are not shared by the AL complex, no attempt was made to compare by serological method the relationship between the AL complex and the human amebae.

Because of the fact that the free-living Hartmannellid amebae (also known as acanthamebae) have been reported as contaminants of monkey tissue culture (10, 22), a preliminary immunologic comparison was made between the AL complex and a Hartmannellid ameba (Lilly strain) originated from a tissue culture (10). Results are summarized in Table II. Rabbits immunized with the Hartmannellid amebae developed high titers of CF antibody against the ameba and lower titer CF antibody against the AL complex. A postimmunization serum from a rabbit immunized with the Hartmannellid ameba was given to us by Dr. C. G. Culbertson for comparative study. Its CF antibody titer

TABLE II
Antibody Titers of Serums from Rabbits Immunized with the AL Complex and the Hartmannellid Ameba (Lilly Strain)

Rabbits immunized with	CF antibody vs.		SEA antibody
	AL complex	Lilly amebae	
AL complex	10/160*	0/10	160/2560
" "	0/40	0/0	40/2560
" "	10/160	0/0	40/2560
" "	10/640	0/40	40/2560
Lilly amebae	0/80	0/320	40/40
" "	0/160	0/640	40/80
Lilly amebae‡	20	250	N.T.

* 10/160 means antibody titer of 1/10 for preimmunization and 1/160 for postimmunization serums; "0" means negative at 1/10; N.T., not tested.

‡ This particular antiserum was supplied by Dr. C. G. Culbertson who made the original isolation of the Lilly strain, and the CF antibody against the Lilly amebae was said to be between 250 and 1000.

against the Hartmannellid ameba was said to be 1/250 to 1/1000. When tested with the AL complex, the titer was only 1/20. Immunization with the Hartmannellid ameba failed to induce a rise in the SEA antibody. Rabbits immunized with the AL complex developed high titer of CF and SEA antibody against the AL complex, but only one of four animals developed a significant titer of CF antibody against the Hartmannellid amebae. Attempts to demonstrate an erythrocyte-sensitizing antigen from the Hartmannellid ameba have been unsuccessful.

In a parallel CF test of serums from 18 adults and 20 children living in the Boston area, 32 were positive for antibody against the AL complex, and only 3 were positive against the Hartmannellid ameba. One may conclude that the AL complex shared antigenic component(s) with, but is distinct from, the Hartmannellid ameba. In this connection, it should be emphasized that rabbits immunized with the AL complex showed a greater than fourfold rise in antibody

against human cells (9). More recent study has shown that three of four guinea pigs immunized with the Lich cells suspended in Freund's adjuvant responded with a greater than fourfold rise in CF antibody against the AL complex. Thus, one also has to conclude that the AL complex shares common antigens with the Lich cells.

DISCUSSION

Evidence has been presented showing that the inoculation of the ameboid cell-"lipovirus" (AL) complex into a culture of human cells (Lich cells) resulted in the development of characteristic changes in the nuclei of some of the human cells. While the nuclei of some Lich cells showed marked abnormalities, the cytoplasm of the same cells remained normal in appearance. This suggests that the primary changes probably occur within the nuclei. Time-lapse cinematography shows also that nuclear shrinkage occurred prior to cytoplasmic retraction (Figs. 4 *a* to 4 *f*). With the removal of the ameboid cells from the inoculum or the spatial separation of the ameboid from the Lich cells by a membrane filter (Millipore, 150 μ in thickness and 0.45 μ in pore size), nuclear abnormality was no longer observed. In a single successful experiment studying the sequential change of a sparsely populated Lich culture inoculated with a few ameboid cells, a Lich cell, after a 33 min intermittent contact with an ameboid cell, was observed to undergo the following changes: it remained polygonal in shape for 33 hr, divided once, became rounded, and remained rounded without rupturing for at least another 40 hr. In the same field of observation, Lich cells that were not in contact with the ameboid cell remained normal in appearance and continued to divide. Based on these observations, it appears reasonable to conclude that the changes which occurred to the Lich cells were not due to toxins released by the ameboid cells, but were the consequence of the transfer of a yet unidentified factor(s) from the ameboid to the Lich cell by an unknown mechanism that required cell-to-cell contact.

To explore the nature of this factor(s), we performed experiments to determine if the optimal development of nuclear lesions require DNA synthesis, if AL antigens can be found within the human cells in an inoculated culture, and if DNA materials can be found in the cytoplasm of the ameboid cells. The results are all in the affirmative. As shown in Table I, the development of nuclear lesions in inoculated Lich culture is partially but significantly suppressed by 5-fluorodeoxyuridine, and this suppression is reversed by thymidine. One can conclude, therefore, that the optimal development of nuclear lesions requires DNA synthesis. The accumulation of AL antigens within human cells is described in the accompanying report (4). While one may find several possible explanations for the presence of AL antigens within the human cells, the most logical one is the synthesis of new antigens as specified by the transferable factor(s). As shown in Figs. 5 *a* and 5 *b*, by radioautographic

technic, thymidine-containing DNase-sensitive materials are found in the cytoplasm of the ameboid cells. Based on the current knowledge of the chemistry of nucleic acids, the thymidine-containing DNase-sensitive materials must be considered as DNA. These findings suggest the possibility that the transferable factor may contain DNA, may have replicated in the human cell, and they specify the synthesis of immunologically specific proteins.

Whether the cytoplasmic DNA of the ameboid cell is the transferable factor(s) remains to be investigated. The possibility that the cytoplasmic DNA may be PPLO was considered. Since no microscopically visible microorganism can be demonstrated in the ameboid cells by cytological and cultural examinations, this possibility was considered unlikely. The transferable factor(s) is probably unrelated to the Kappa particles of the paramecium. The Kappa particles are said to be as large as the rickettsiae and bacteria, and are visible under a light microscope (23).

One obvious question is whether the ameboid cell of the AL complex is an ameba, and whether amebae contain similar transferable factors. This possibility is being investigated. If one defines an ameba as a unicellular organism which has a small ring-shaped nucleus (Fig. 2 *h*) and which manifests ameboid movement, then the ameboid cell is an ameba. Results of preliminary serologic study with relatively crude tests reveal distinct antigenic overlapping between the AL complex and the Hartmannellid ameba (Lilly strain) and between the AL complex and cultured human (Lich) cells.

Whether human cells which have accepted the transferable factor(s) from the AL complex may occasionally transform into ameboid cells is also a question that requires further study. This remote possibility is suggested by the formation of DNA rings around the nucleoli of affected human cells (Figs. 2 *e*, 3 *c*, and 4 *f*) and the superficial similarity of such ring-structure to the nuclei of the ameboid cell (Figs. 2 *f* and 3 *d*). The discrepancy between the actual increase in the number of tissue culture units of AL complex and the expected increase calculated from the frequency of binary divisions of the ameboid cell in an inoculated Lich culture is a finding that requires investigation. The discrepancy may be due to the fact that the field monitored by the time-lapse cinematogram is not representative of the whole culture, or due possibly to the appearance of ameboid cells by mechanism other than binary divisions of preexisting ameboid cells.

In a recent communication (24) Dunnebacke described plaque formation on monolayers of chick embryonic tissue cultures inoculated with the AL complex. Using micromanipulation to isolate single cells, she was able to show that each ameboid cell yielded one plaque. Each plaque was composed of many ameboid cells and some degenerated tissue cells. The content of a ruptured ameboid cell was unable to initiate plaque formation. There were, however, rare exceptions. Occasionally, the content of a singly ruptured ameboid cell yielded mul-

tiple plaques. She did emphasize her recent difficulty in recovering a plaque-forming agent from the content of singly ruptured ameboid cells. Thus, the significance of this communication in relation to the possible existence of a subcellular agent capable of transforming a human cell into an ameboid cell remains unclear. We are of the opinion that, at this time, there is no direct evidence to support the hypothesis that an occasional human cell may be transformed into an ameboid cell. This possibility, however, deserves further investigation. The two preliminary papers (9, 25), in which the AL complex was referred to as "lipovirus"-altered human cells must be reinterpreted in light of the present findings.

The relation of the AL complex to man remains obscure. An earlier report (16) of the high prevalence of antibody against the AL complex in man has been confirmed by a more extensive seroepidemiologic study. With improved antigens, 80 to 100% of children and young adults residing in Boston, Hong Kong, and Tunisia were found positive for antibody against the AL complex. Seroconversions of several babies in the Boston area have also been observed to occur at 8 to 12 months of age. It is not known at this time whether the antibody in man is against the "lipovirus," the ameboid cells, the AL complex, or a common human pathogen serologically related to the AL complex. Results of an extensive seroepidemiologic study of the AL complex will be presented shortly.

Repeated attempts to reisolate a similar agent from bloods of hepatitis patients have been unsuccessful. It is very likely that the relation to hepatitis in the original isolation was purely fortuitous, or possibly, bloods are poor sources of the AL complex. Of interest is the recent report of Pereira, Marsden, Corbitt, and Tobin on the isolation of 6 strains of Ryan virus from patients with respiratory or generalized infection (26). Based on the reported characteristics of the Ryan virus, it is possible that the Ryan virus and the AL complex may be related.

SUMMARY

Experiments designed to elucidate the nature of the "lipovirus" are described. The development of characteristic nuclear lesions in human cells *in vitro* depended on the presence of an ameboid cell in the inoculum. The spatial separation of the ameboid cells from the human cells by a membrane filter 150 μ in thickness was sufficient to prevent the development of nuclear lesions. Nuclear lesions appeared to be the primary change of the affected human cells. This development of nuclear lesions was partially suppressed by FUDR and the suppression was reversed by thymidine.

Time-lapse microcinematography showed that a 30 min intermittent contact between an ameboid cell and a human cell resulted in the retraction of both progenies of the human cell after a lapse of about 36 hr. Other human cells not in contact with the ameboid cell remained polygonal and continued to divide.

Radioautography of the ameboid cell revealed the presence in the cytoplasm of thymidine-containing DNase-sensitive materials.

The development of antigens related to the ameboid cell within the cytoplasm of the human cell is described in the accompanying report (4).

We are grateful to Doctors Robley C. Williams, Thomas C. Frothingham, Elmer Pfefferkohn, and Steve C. Pan for their helpful suggestions in the preparation of this manuscript, to Dr. John C. Snyder for his continuing interest in this work, and to Dr. Robert P. Geyer for his help with time-lapse microcinematography.

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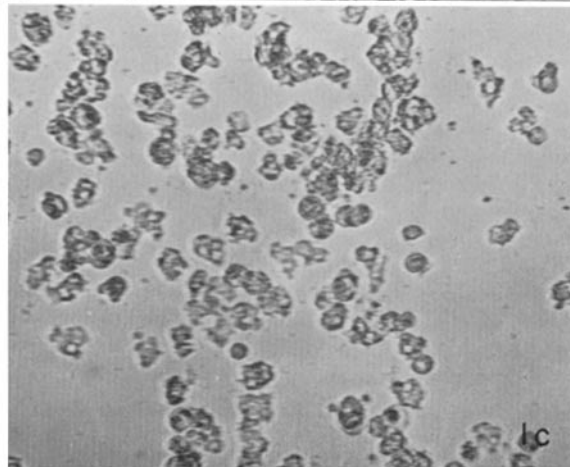
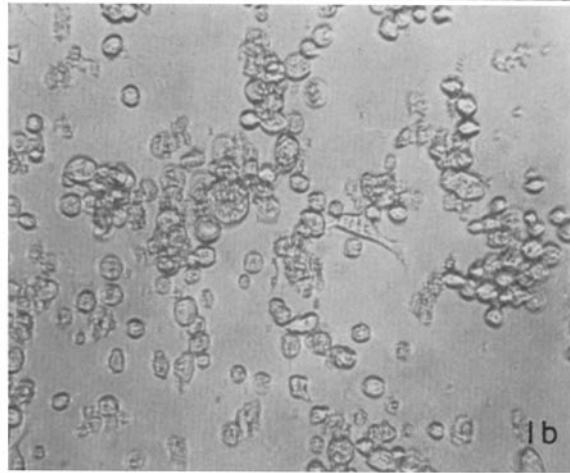
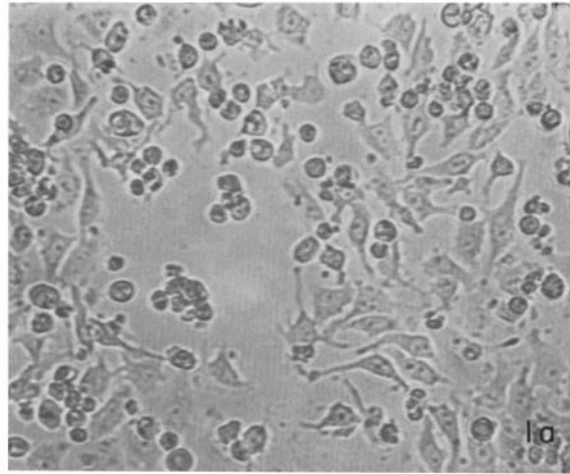
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EXPLANATION OF PLATES

PLATE 121

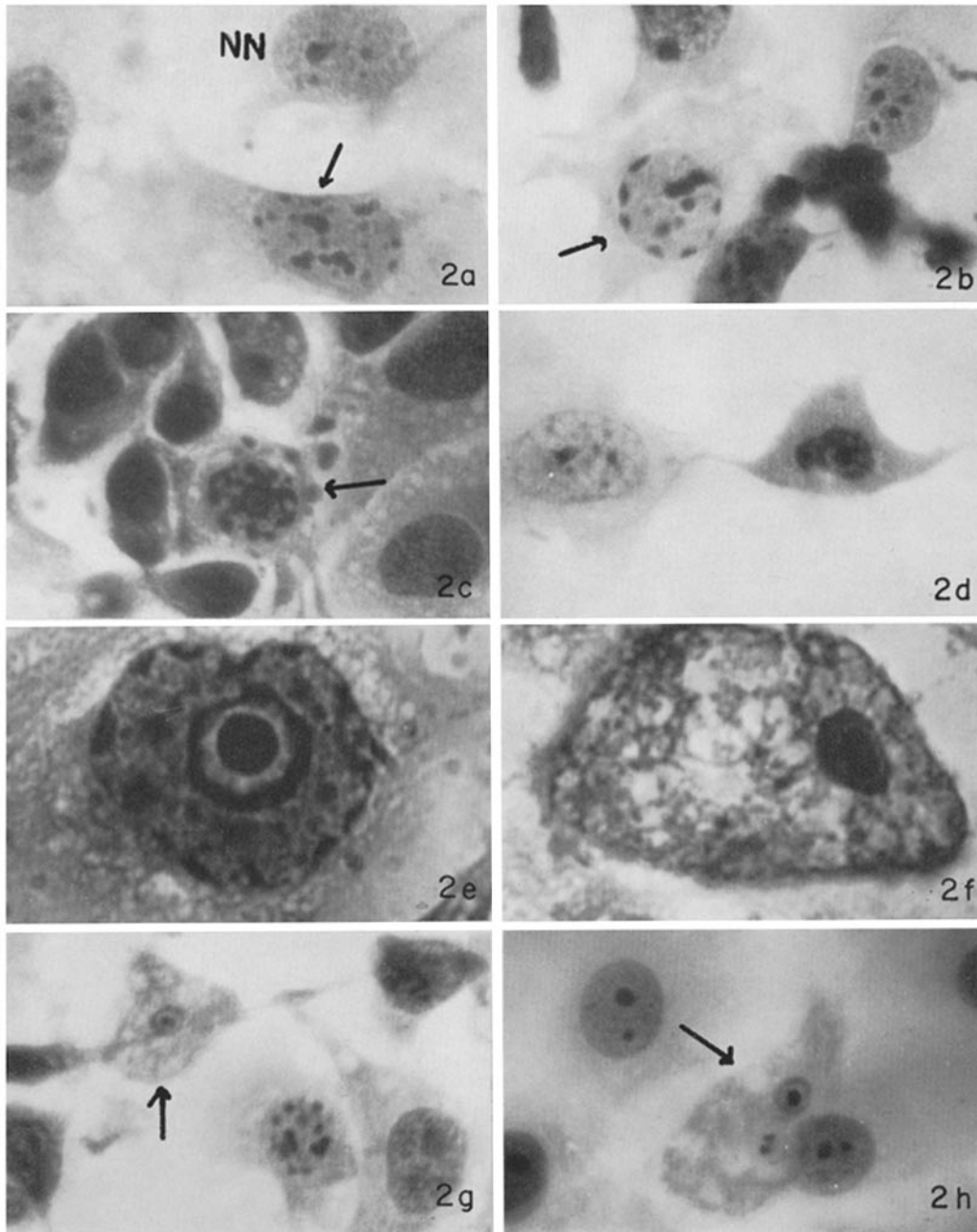
FIGS. 1 *a* to 1 *c*. Cytopathic changes in Lich culture inoculated with AL complex; unstained, $\times 100$. (Fig. 1 *a*) An early focus consisting chiefly of some rounded granular cells among the normal ones; (Fig. 1 *b*) advanced changes consisting of round cells and bizarre-shaped cells; and (Fig. 1 *c*) complete change in which the entire Lich culture was replaced by the bizarre-shaped cells.



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PLATE 122

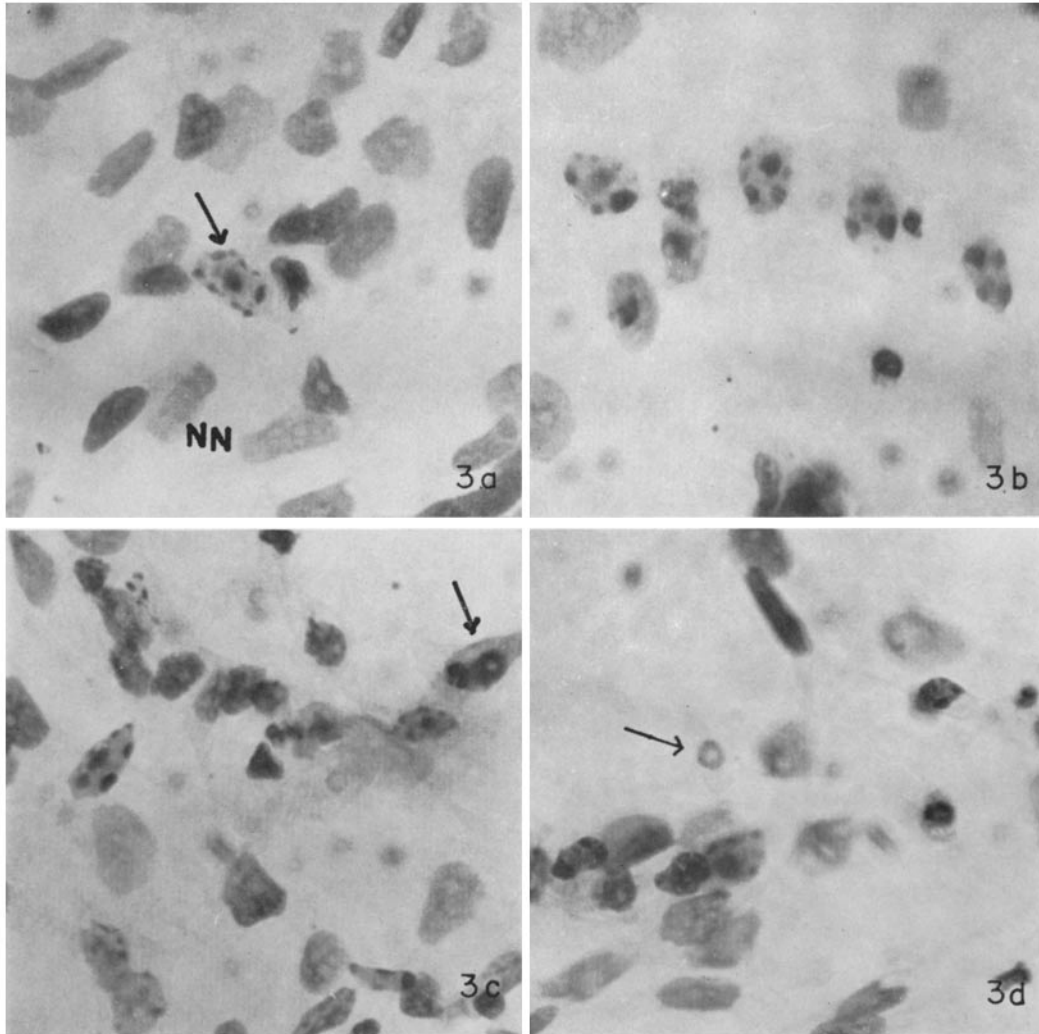
FIGS. 2 *a* to 2 *h*. Nuclear changes in Lich culture inoculated with the AL complex (fixed with Bouin's fluid and stained with acid hematoxylin). (Fig. 2 *a*) A Lich cell with normal nucleus is indicated by *NN*, and arrow points to a cell with basophilic clumps, $\times 400$. (Fig. 2 *b*) "Margination of basophilic material, $\times 400$. (Fig. 2 *c*) Ring formation of basophilic material around the nucleolus, $\times 400$. (Fig. 2 *d*) A Lich cell with shrunken or "pyknotic" nucleus, $\times 400$. (Fig. 2 *e*) Nucleus of a Lich cell with a prominent nucleolus surrounded by a basophilic ring, $\times 1000$. (Fig. 2 *f*) An ameboid cell, $\times 1000$; note the absence of visible cytoplasmic parasite and some resemblance of its nucleus to that of the Lich cell in E. (Fig. 2 *g*) An ameboid cell with its characteristic nucleus, $\times 400$. (Fig. 2 *h*) A mixed culture of monkey kidney cells and Hartmannellid amebae, $\times 400$; it was photographed from a fixed preparation kindly furnished by Dr. C. G. Culbertson. Nuclei of the monkey kidney cells in this slide did not show any of the abnormalities described in Figs. 2 *a* to 2 *e*.



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PLATE 123

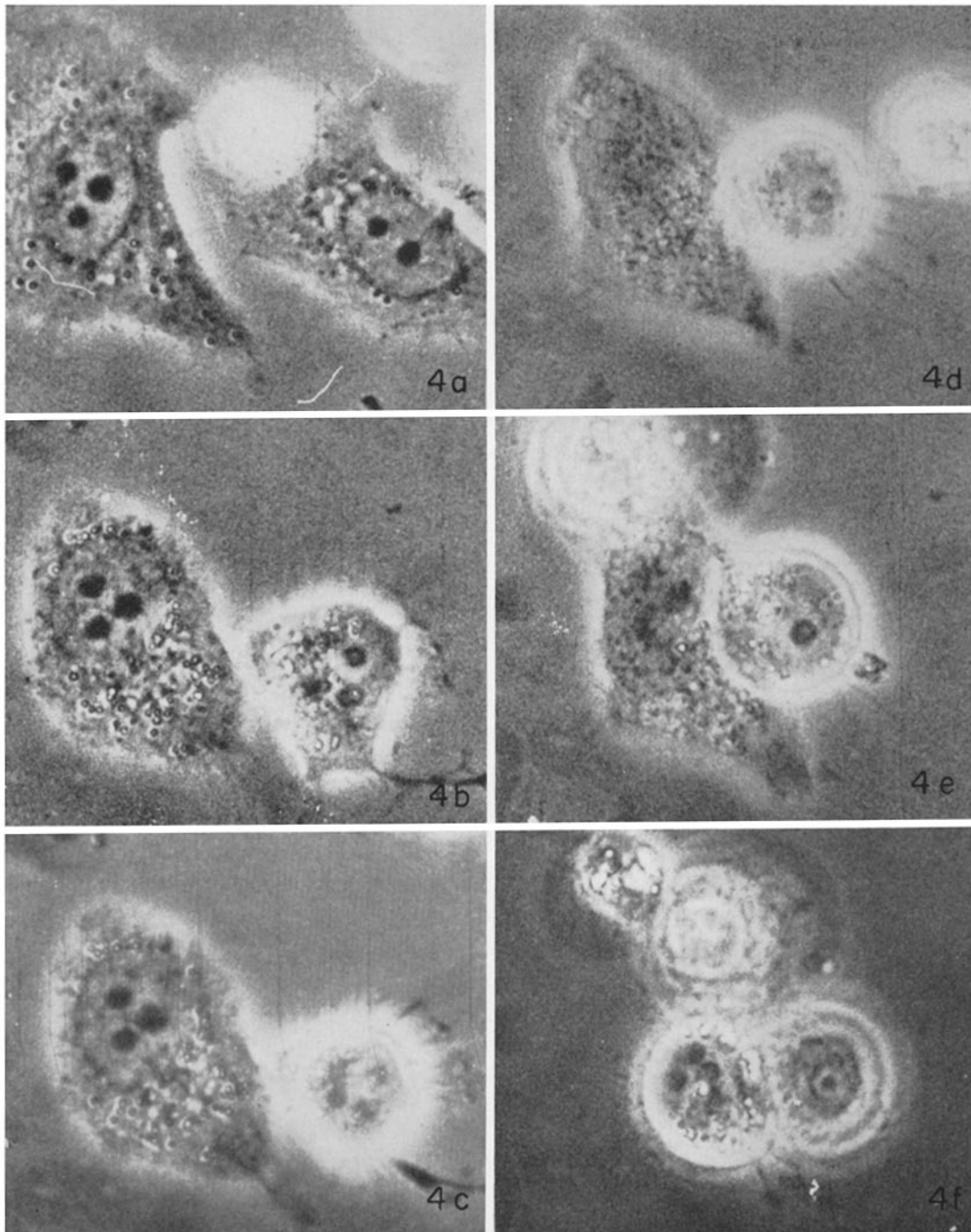
FIGS. 3 *a* to 3 *d*. Nuclear changes in Lich culture inoculated with AL complex; fixed with Bouin's fluid and stained with Feulgen's, $\times 400$. These photomicrographs show that nuclear changes involved primarily Feulgen positive material. (Fig. 3 *a*) Normal nucleus (*NN*); and abnormal nucleus (arrow) with "margination" of Feulgen-positive material. (Fig. 3 *b*) Abnormal nuclei with large prominent masses of Feulgen positive materials. (Fig. 3 *c*) Formation of Feulgen-positive ring within the nucleus of a Lich cell. (Fig. 3 *d*) The nucleus of an ameboid cell consisting of a Feulgen-positive ring which resembles those shown in Fig. 3 *c*.



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PLATE 124

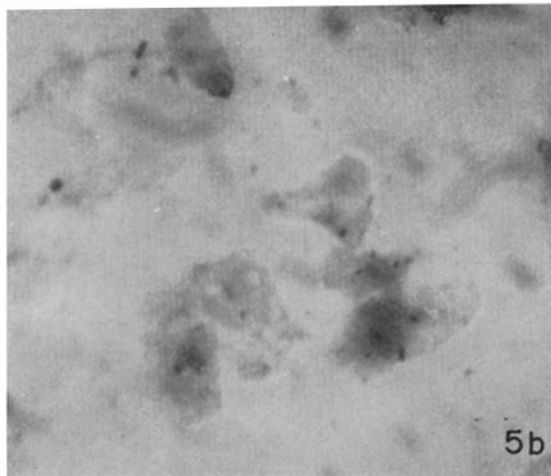
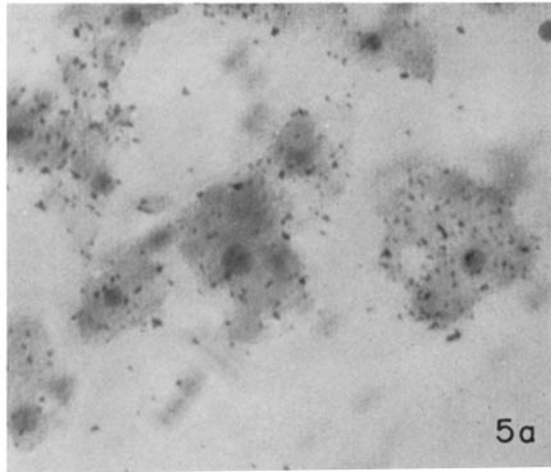
FIGS. 4 *a* to 4 *f*. Time-lapse cinematography of inoculated Lich culture showing the sequential changes in 2 Lich cells; phase contrast, $\times 560$. The whole sequence lasted 22 hr (the interval between Fig. 4 *a* and Fig. 4 *b* was 7 hr; between Fig. 4 *b* and Fig. 4 *c*, 2 hr; between Fig. 4 *c* and Fig. 4 *d*, 4.6 hr; between Fig. 4 *d* and Fig. 4 *e*, 4.5 hr; and between Fig. 4 *e* and Fig. 4 *f*, 4.5 hr). Note the shrinkage of the nucleus which was followed by rounding of the whole cell. The "nucleus" of the round cell in Fig. 4 *f* is remarkably similar to the nucleus of the ameboid cell. Note also the absence of constant prodding by the bizarre-shaped cells.



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PLATE 125

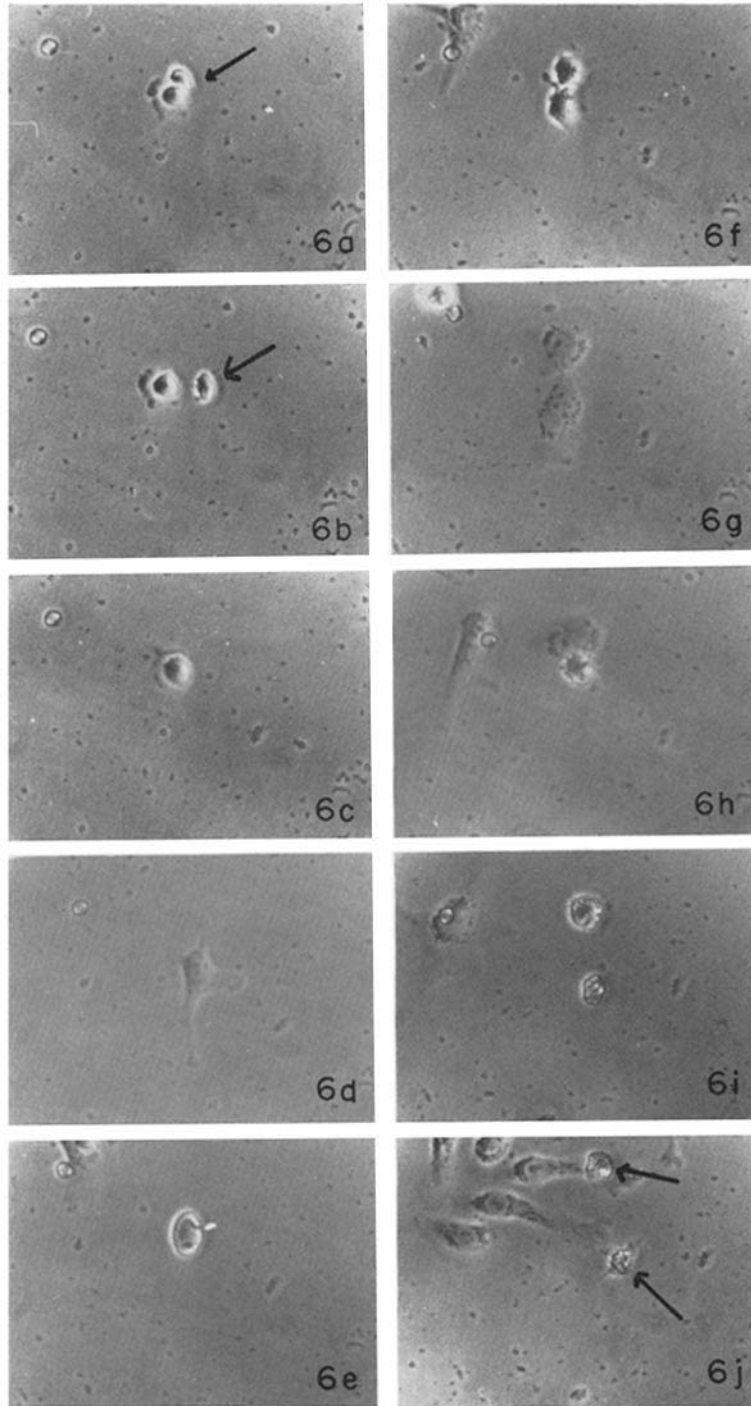
FIGS. 5 *a* and 5 *b* Radioautographs of the AL complex labeled with thymidine- H^3 , $\times 400$. (Fig. 5 *a*) Note the concentration of grains over cytoplasms of the ameboid cells, the variation in grain number per cells, and the unexplained absence of grains over nuclei. (Fig. 5 *b*) Similar preparation as Fig. 5 *a* but predigested with DNase; note the virtual absence of grains over the ameboid cells.



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PLATE 126

FIGS. 6 *a* to 6 *j*. Fate of a single Lich cell after contact with an ameboid cell as recorded by time-lapse cinematography (magnification of 100, 20 sec per frame). (Fig. 6 *a*) Contact of the Lich and ameboid (arrow) cell. (Fig. 6 *b*) Ameboid cell (arrow) moved away after about 33 min of intermittent contact (no ameboid cell was seen to move into the field of observation hereafter). (Fig. 6 *c*) The single Lich cell 5 hr later. (Fig. 6 *d*) The Lich cell now assumed its characteristic "epithelial-like" appearance (14.2 hr after Fig. 6 *c*). (Fig. 6 *e*) In another 9.7 hr, the Lich cell rounded up for division (note also the first appearance of a portion of a Lich cell moving into the upper left corner; this was a result of the division of several Lich cells situated just outside the field encompassed by the movie but well within the field of observation of the microscope). (Fig. 6 *f*) Division completed in 68 min. (Fig. 6 *g*) Two daughter cells with normal appearance. (Fig. 6 *h*) Retraction of one daughter cell (6.6 hr after Fig. 6 *f*). (Fig. 6 *i*) Retraction of the other daughter cell 6.5 hr after Fig. 6 *h*. (Fig. 6 *j*) 13 hr after Fig. 6 *g*, the two daughter cells (arrows) remained as rounded masses while more normal Lich cells migrated into the field. The experiment was terminated 35 hr later. During this interval, the two retracted cell masses remained unchanged while the other normal-appearing Lich cells continued to divide and extend further into the field.



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