IMMUNOFLUORESCENT STUDIES OF HUMAN CELL CULTURES AND CHICK EMBRYOS INOCULATED WITH THE AMEBOID CELL–"LIPOVIRUS" COMPLEX*, ‡

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Plates 127 to 129

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The nature of the ameboid cell–"lipovirus" (AL) complex has been described in the preceding paper (1). This paper describes the development of antigen in human cell cultures inoculated with the AL complex, and the pathogenicity of the AL complex for chick embryos.

Materials and Methods

Ameboid Cell–"Lipovirus" Complex.—A seed of AL complex grown in chick embryo tissue cultures was received from Dr. Robert Chang of the Harvard School of Public Health. This seed was passed into HeLa tissue cultures in our laboratory to prepare a stock inoculum for subsequent experiments and immunization of rabbits, and is referred to as the HeLa seed. The tissue culture units of various passages ranged from 4.2 to 5.3 $\log_{10}$/0.1 ml when titrated in HeLa cells.

Another "purified" seed was received from Dr. Chang. The method of "purifying" this seed inoculum by terminal dilution has been described (1). This seed was passed in human liver (Lich) cells to make a stock inoculum and stored at 4°C. This stock inoculum had a tissue culture unit titer of 4.5 $\log_{10}$/0.1 ml and is referred to as the Lich seed.

Tissue Cultures and Chick Embryos.—All tissue culture cells were prepared in our own laboratory. The HeLa cell line has been in our laboratory for many years. The human liver cells (Lich) were originally obtained from Dr. Chang. The continuous line of human amnion cells (AV_3) was originally obtained from Dr. Rachel Mason of the Charles Pfizer Company, Terre Haute, Indiana and has been used in our laboratory for the last 5 yr for studies of measles virus.

The growth medium for HeLa cells was Eagle's + Earle's media with 15% inactivated human serum. For maintenance, a medium consisting 70% M199, 25% tryptose phosphate broth, 5% inactivated calf serum and 0.2% dextrose with a pH adjust to 7.4 was used.

The growth and maintenance media for Lich cells consisted of modified Eagle's basal medium with 5% and 3% inactivated calf serum, respectively (2).

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The growth and maintenance media for AV_3 cells were similar to that described previously (3).

Antibiotics consisted of 1000 units of penicillin, and 1000 μg streptomycin per milliliter were incorporated in all tissue culture media.

Chick embryos of 10 or 13 days of age preincubated in a local hatchery were furnished to us. After inoculation for experiments, they were incubated at specified temperatures either at 32° or 37°C.

Inoculation and Harvest of Specimens.—For Leighton tube experiments, 150,000 HeLa, 75,000 Lich or 150,000 AV_3 cells were planted and incubated for 4 to 6 days before inoculation. The AL complex inoculum for tissue culture experiments was usually 1000 to 10,000 TCD_{50} units in 0.1 ml of volume. After inoculation, the Leighton tubes were returned to incubators. 2 hr after inoculation, the cultures were rinsed twice with 0.01 M phosphate-buffered saline (PBS: pH 7.0) and replaced with fresh media. As controls, similar tubes were inoculated with 0.1 ml of maintenance media and treated similarly. At periodic intervals, two inoculated tubes and one control tube were harvested. The cover slips were taken out, rinsed twice in PBS, and air-dried. After drying, they were immediately fixed in acetone for 10 min, dried, and stained with fluorescent conjugate. If fluorescent staining was not possible on the same day of harvest, the acetone-fixed cover slips were stored in a refrigerator at 4°C until processed. The media from the two inoculated tubes were pooled and stored at 4°C until HeLa cell cultures were available for titrations.

In chick embryo experiments, 0.2 ml containing approximately 1000 to 10,000 tissue culture units of AL complex was inoculated either allantoically or amniotically. Control embryos were inoculated in the same manner with tissue culture media. Inoculated embryos were placed in incubators with a temperature of 32°C. In one experiment, half of the inoculated embryos were incubated at 32°C and the other half at 37°C to observe the temperature effect. Inoculated embryos were observed daily by candling for viability. On different days after inoculation, the chorio-allantoic membrane, the amniotic membrane, and the embryo were harvested, placed in test tubes, and immediately frozen in dry CO_2-ethanol mixture. After freezing, the specimens were kept at −65°C for future frozen sectioning. The allantoic-amniotic fluids were pooled and kept at 4°C for titrations. In one experiment, individual organs of the chick embryo such as the brain, lung, liver, stomach, and intestine were harvested and divided for frozen sectioning and infectivity titrations.

Preparation of Anti-AL Complex Fluorescent Conjugate.—Rabbits weighing about 5 to 6 pounds were given seven weekly intravenous injections of 2,000,000 AL complex-inoculated HeLa cells. 1 wk after the last injection, rabbits were bled by cardiac puncture. Serums were separated by centrifugation and pooled. After precipitation with half saturated ammonium sulfate, the globulin fraction was labeled with fluorescein isothiocyanate (4). The fluorescent conjugate was absorbed with mouse liver powder before use for direct fluorescent staining (5).

Preparation of Specimens for Fluorescence Microscopy.—6-μ-thick frozen sections of harvested chick embryos were cut in a cryostat according to the method of Linderstrøm-Lang and Mogensen (6) as modified by Coons et al. (7). For whole embryos, sections were cut at different levels through the brain, the lung, and the liver. After acetone fixation, the sections were ready for fluorescent antibody (FA) staining. Staining time for the indirect method was 30 min for unlabeled rabbit anti-AL complex serums, 10 min of rinsing with PBS, and 30 min of anti-rabbit sheep globulin conjugates. For direct method, the staining time was 30 min. Both procedures were carried out at room temperatures. After 10 min PBS rinsing, slides were mounted under a cover slip with buffered glycerin at pH 7.0.

A Leitz ortholux fluorescence microscope with monocular eyepiece, exciting filter of Corning 5840, and barrier filter of Wratten 2A was used for examination of slides. A special beam-splitter attachment was installed to allow simultaneous observation with fluorescence and

phase-contrast microscopy. Kodak Tri-X black and white film (ASA-400) or Kodak daylight Ektachrome (ASA 100) was used for photography.

EXPERIMENTAL RESULTS

Comparison of Direct and Indirect FA Staining

Inoculated tissue culture cells and chick embryo sections showed specific fluorescence when stained by either the direct or indirect method. Detailed description of the morphology of such cells will follow. When tested in liver section from chick embryos inoculated with the AL complex, the preinoculation rabbit serum showed no specific staining at 1/10 dilution while the postinoculation serum showed specific fluorescent staining in serial 2-fold dilutions up to 1/640 or greater. This rise in fluorescent staining titer was also reflected by a

TABLE I

Antibody Titers in Rabbit Serum Immunized with AL Complex

Serum	FA titer		CF titer
	Direct	Indirect	
Preinoculation	nd	<10	<10
Postinoculation	80*	≥640	160

nd, not done.

* Reciprocal of serum dilution.

rise of complement fixation tests performed according to the method as described by Chang (8) (Table I). When the globulin fraction of postinoculation serum was labeled with fluorescein isothiocyanate and used for direct FA staining, specific fluorescent staining was obtained up to a dilution of 1/80. In general, the indirect FA method showed more background fluorescence on noninfected cells while the direct staining showed practically no background fluorescence at all even at a dilution of 1/10. Delineation of the morphology of infected cells was also more distinct by the direct method. Since the direct method is simpler to perform and gives better morphological visualization, it was chosen for subsequent studies.

Specificity of the Fluorescent Staining

The specificity was determined by three methods: (*a*). No specific fluorescence was seen in control chick embryo sections or tissue culture cells when stained by the AL complex conjugate. (*b*). There was no inhibition of specific fluorescent staining by preinoculation rabbit serum at 1/10 dilution, but complete inhibition was effected by postinoculation AL complex serum up to 1/80 dilution (Table II). (*c*). No specific fluorescence was seen in AL complex–inocu-

lated tissue cultures or chick embryo sections when stained with heterologous conjugates (Table III).

Observations of Tissue Cultures Inoculated with the AL Complex

A. *Inoculation with Unfrozen Suspensions of AL Complex.*—Both the HeLa seed and the Lich seed of AL complex were used to inoculate Leighton tube

TABLE II

Inhibition Tests of FA Staining on Cells Inoculated with AL Complex

Method: 1. Frozen sections were treated with unlabeled anti-AL complex serum for 18 hr at 4°C.
2. Rinsed with PBS, and stained with anti-AL complex conjugate for 15 min at room temperature.

	Serum dilution					
	1/10	1/20	1/40	1/80	1/160	1/320
Preinoculation	++++*	++++	++++	++++	++++	++++
Postinoculation	0	0	0	0	0	++
Control (direct staining)	++++					

* Degree of fluorescent staining. ++++, maximum, no inhibition; 0, none, complete inhibition.

TABLE III

Test for Cross-Reactions on AL Complex with Heterologous FA Conjugates

Conjugates against	FA staining
AL complex	++++
Parainfluenza-2	0
Parainfluenza-3	0
Herpes simplex	0
Western equine encephalitis	0
West Nile	±
Influenza A_2	0
Theiler's GD VII	±

tissue cultures of HeLa, AV_3, or Lich cells. In various experiments, the sequential morphological changes in inoculated culture cells were similar, with increasing number of foci showing specific fluorescence as the experiment progressed. The following description is from an experiment of inoculating 50,000 tissue culture units of Lich seed of AL complex in HeLa cells, making an inoculum-cell ratio approximately 1/10. To record the actual increase of specific fluorescent foci, the inoculated cell sheet on the cover slip harvested at different time inter-

vals was scanned under a fluorescence microscope with a 10 × eyepiece and a 10 × objective. For counting a cover slip with small number of fluorescent foci, the entire cover slip of 35 × 10 mm area or 140 microscopic fields were scanned. For cover slips with large numbers of fluorescent foci, approximately 30 microscopic fields were examined and the number was multiplied by a ratio of 4.7 to obtain the total FA foci number per cover slip. Serial 10-fold dilutions of harvested tissue culture fluids were inoculated in HeLa cell cultures and in-

TABLE IV

Correlation of Cytopathogenic Effect (CPE), Increase in Fluorescent Foci Number, and Tissue Culture Units in HeLa Cells Inoculated with Lich Seed of AL Complex

Time of harvest	CPE		FA foci No./350 mm²		Tissue culture units $\log_{10}TCD_{50}$/0.1 ml	
	A*	B	A	B	A	B
hr						
0.1	0	0	197	0	3.0	1.7
2	0	0	785	0	nd	1.7
4	0	nd	644	0	2.2	nd
8	nd	0	nd	0	nd	0.7
18	0	nd	2,195	nd	3.7	nd
24	0	0	2,100	0	3.2	0.5
32	nd	0	nd	0	nd	0.6
48	+++‡	0	56,400	1	4.0	0.7
72	++	0	59,200	0	4.5	2.0
96	+++	0	84,600	24	4.5	2.5
120	nd	0	nd	220	nd	3.0
140	nd	+	nd	190	nd	3.0

nd, not done.

* A, inoculum of 5×10^4 $\log_{10}TCD_{50}$ AL complex; B, inoculum of 10^3 $\log_{10}TCD_{50}$ AL complex.

‡ +++, marked cytopathic effect; +, minimal cytopathic effect.

cubated at 32°C. Inoculated tubes were observed for 14 days for cytopathic changes. The titer of tissue culture units was calculated according to the method of Reed and Muench (9). Experimental results are presented in Table IV and Text-fig. 1.

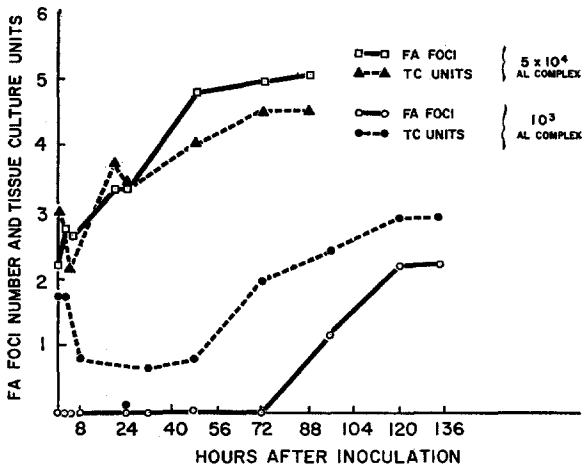
It can be seen that the number of fluorescent foci increased markedly as the experiment progressed. By 48 to 72 hr after inoculation, the number had increased to more than 250-fold over that at the beginning of the experiment. However, the increase in tissue culture units was only 1.5 log, 10 times less than the actual increase of fluorescent foci number. The explanation of this discrepancy was unclear, since similar discrepancies were seen in other experiments; e.g., inoculation of Lich seed in Lich or AV_3 cells or inoculation of HeLa seed in HeLa, Lich, or AV_3 cells.

Morphological observations of inoculated cell sheets stained with AL complex fluorescent conjugate are described as follows.

Before inoculating the Leighton tubes, a smear from the Lich seed suspension was made on a slide and stained with the anti-AL complex FA conjugate. Crenated round cells with intense homogeneous fluorescence were seen (Fig. 1).

At 5 min after inoculation, cover slips harvested from Leighton tubes showed a number of crenated fluorescent cells in different areas indicating probably the original inoculum was dispersed on the cell sheet.

At 2 hr, just before the cover slips in Leighton tubes were washed and replaced with new



TEXT-FIG. 1. Number of fluorescent foci and tissue culture units of AL complex in cultures of HeLa cells inoculated with the AL complex.

media, the number of fluorescent cells was about the same as seen in the 5 min harvest. However, in some of the areas, a few neighboring cells in contact with the original crenated cells began to show granular fluorescence in their cytoplasm.

At 4 hr (2 hr after washing and medium replacement), the number of fluorescent foci characterized by a central cell with homogeneous fluorescence surrounded by several neighboring cells with granular punctate fluorescence was definitely more apparent (Fig. 2) suggesting that the central cell was spreading a transmissible factor or factors to the neighboring cells.

At 18, 24, 32, and 48 hr, examinations of the harvested cover slips showed definite increase in the number of fluorescent foci (Text-fig. 1). Many cells showed punctate granular fluorescence in the cytoplasm with nuclei entirely devoid of fluorescent antigen (Fig. 3). When these cells were examined by phase-contrast microscopy, the morphology of the chromatin pattern and the morphology of the nucleoli were indistinguishable from those cells which did not contain AL complex antigen (Figs. 7 to 12).

At 72 to 96 hr, the number of fluorescent cells was greatly increased to more than 250-fold in comparison with the beginning of the experiment. Most cells now showed intense cytoplasmic fluorescence, multiple cytoplasmic vacuoles, and ciliated protrusions from the cell surface (Fig. 4). Under phase-contrast microscopy, the nucleus was small and not readily discernible. Eventually almost all cells assumed the shape of a bizarre, round, and crenated appearance.

At a low multiplicity, when 1000 tissue culture units of Lich seed were inoculated, no fluorescent focus was detectable on the cover slips until 96 hr after inoculation (Table IV and Text-fig. 1). The appearance of these foci was similar to that seen in the early period of the previous experiment where one crenated fluorescent cell was surrounded by several cells of normal nuclear morphology with fine cytoplasmic fluorescent granules (Figs. 13 and 14). At the same time, a definite increase in the number of AL complex tissue culture units was also observed. When only 10 tissue culture units were given, no detectable fluorescent focus was encountered during an entire observation period of 140 hr. Therefore, the increase in fluorescent foci was dose dependent.

B. *Inoculation with Frozen and Thawed AL Complex Preparations.*—A Lich seed containing approximately 1,000,000 cells was frozen and thawed 10 times alternately in a dry CO_2-ethanol mixture and a 37°C water bath. The frozen-thawed preparation was centrifuged at 2000 RPM for 15 min. The supernatant was used for inoculation to 40 Leighton tubes of Lich cells. An aliquot of untreated Lich seed was also inoculated to similar tubes as controls.

The Leighton tubes inoculated with frozen-thawed AL complex were divided into 2 groups. Group A tubes were nourished with standard maintenance medium (modified Eagle's medium + 5% calf serum) and Group B tubes were nourished with an enriched medium (modified Eagle's medium + 10% calf serum + 0.5% yeast extract and 0.5% casein hydrolysate). The media were changed twice a week. Tubes with cover slips were harvested for immunofluorescent staining and the fluid was saved for titrations as in previous experiments.

For the first 6 days, fluorescent specks were seen in the cytoplasm of Lich cells. These fluorescent specks were larger and more irregular in shape than the fluorescent granules seen in experiments inoculated with unfrozen AL complex (Fig. 15). They distributed randomly over the cell sheet and did not have any organized pattern. About 60 to 70% of cells on such cover slips showed the presence of such fluorescent specks. Between the 7th and 16th days of harvest, these fluorescent specks were still detectable in the cells but the number of cells containing such fluorescent specks decreased gradually as time went on. At no time throughout the experimental period was there any presence of bizarre fluorescent ciliated cells as seen in controls. Titrations also failed to detect AL complex activity in harvested tissue culture media.

Observations of Chick Embryos Inoculated with the AL Complex

A. *Increase of AL Complex Activity in Embryonic Fluids.*—A group of 10-day-old chick embryos were inoculated into the allantoic sac with approximately 1000 tissue culture units of AL complex and incubated at 32°C. At daily intervals, pooled amniotic and allantoic fluids from 3 to 7 eggs were harvested from individual eggs. Titrations were performed by inoculating 2-fold serial dilutions of these fluids into HeLa cell cultures (Table V). It can be seen that, although the mean titers increased as days of experiment progressed, there was marked variation among individual embryo in its content of AL complex.

B. *Effect of Embryo Ages, Route of Inoculation, and Temperature of Incubation.*—60 10-day-old and 60 13-day-old chick embryos were used for this experiment. Half of each group (30 embryos) were inoculated amniotically with 1000 tissue culture units of AL complex and the other half allantoically. Inoculated embryos were again divided into two groups at random (15 embryos in each group) to be incubated at 32° or 37°C. A number of embryos of the same age inoculated with normal tissue culture media were kept in the same incubators to serve as controls. Embryos that died during the first 3 days following inoculation were discarded. Approximately equal number of surviving embryos were harvested daily on the 4th, 5th, and 6th days after inoculation. Embryos found dead or appeared sluggish by candling were harvested first. Since some embryos

TABLE V

Multiplication of AL Complex in Chick Embryos Following Allantoic Inoculations

Days after inoculation	Number of tissue culture units per 0.1 ml of allantoic-amniotic fluids	
	Individual embryos	Mean
1	2*, 1, <1	1
2	1, 16, 12, <1	7
3	32, 160, <1	64
4	128, 128, 256, 2	128
5	57, 64, 12, 2, 128, 128	65
6	212, 64, 212, 1384, 2	371

* Reciprocal of fluid dilutions.

in the control groups also died or became sluggish particularly when incubated at 32°C, the cause of death or sluggishness in inoculated embryos had to be ascertained. Amniotic and allantoic fluids from each harvested embryo were saved for AL complex titration and the amniotic membranes, allantoic membranes, and embryos were frozen for sectioning and examined by FA staining with anti-AL complex conjugate. The results are summarized in Table VI.

From the data in Table VI, it is apparent that 10-day-old embryos when inoculated amniotically and incubated at 32°C were most susceptible to AL complex. This is reflected both in the number of embryos, 10 out of 13, showing AL complex antigen in their tissues and a higher mean AL complex titer in the embryonic fluids.

C. *Appearance of AL Complex Antigen in Embryo Tissues.*—Frozen sections of amniotic membrane, allantoic membrane and cross-sections of embryos at levels through the brain, lung, liver, and intestines were cut. Localization and appearance of antigen seen in chick embryo tissues were not unlike those as described in tissue culture cells. This consisted of scattered foci each made up of 4 to 5 infected cells with granular or homogeneous yellow green cytoplasmic

fluorescence. In embryonic membranes, fluorescent cells were localized mainly in the area beneath the superficial layer of epithelial cells. In chick embryo sections, the liver appeared to be the organ most heavily involved. As few as 1 or 2 to as many as 10 to 20 fluorescent foci may be seen under a low power

TABLE VI

Effect of Age of Embryos, Route of Inoculation, and Temperature of Incubation Inoculated with AL Complex in Chick Embryos

Embryo age	Inoculation route	Incubation temperature	Infection rate	Mean fluid titer $log_{10}TCD_{50}$/0.1 ml
days		°C		
10	Amniotic	32	10/13* (4D, 3S)‡	3.2
		37	0/13	2.4
	Allantoic	32	1/8 (1D, 3S)	2.5
		37	0/12	0
13	Amniotic	32	1/14 (2D, 4S)	1.5
		37	0/9	1.2
	Allantoic	32	0/14 (1D, 3S)	1.5
		37	0/14	0.6

* Numerator, number infected as proved by FA examination; denominator, number harvested between day 4 and day 6.

‡ D, number of embryos died; S, number of embryos appeared sluggish.

TABLE VII

Titration of AL Complex in Tissues of Inoculated Chick Embryos

Tissues	$Log_{10}TCD_{50}$/0.1 ml	FA examination
Embryonic fluids	3.0	nd*
CA-am. mem.‡	2.7	0
Lungs	3.3	+
Liver	2.5	+
Stomach-gut	<0.5	+
Brain	2.7	nd

* Not done.

‡ Chorioallantoic-amniotic membranes.

microscopic field. Both the hepatic parenchymal and hemopoietic cells may be involved (Figs. 5 and 6). In the lung, antigen was seen in interstitial cells. The epithelial cells of the parabronchi were not involved. In the intestines, fluorescent cells were seen in intestinal walls and rarely in the mucosa (Fig. 16). In the brain, groups of neurons with cytoplasmic fluorescence were seen (Fig. 17).

D. *Titration of Inoculated Chick Embryo Tisues for AL Complex.*—10-day-old chick embryos were inoculated with 1000 tissue culture units of HeLa seed

amniotically and incubated at 32°C. On the 5th day after inoculation, most embryos were dead. The embryonic fluids, membranes, lungs, livers, stomachs, intestines, and brains from 3 embryos were harvested. Portions of harvested organs were examined by FA staining and the remaining portions of each organ from these embryos were pooled and titrated in HeLa tissue cultures. The results are shown in Table VII.

It can be seen that all tissues except the stomach and intestines contained significant amounts of AL complex by titrations in tissue cultures and by FA examinations. AL complex antigen was seen in the intestinal wall but titration showed the amount of AL complex activity was less than 0.5 log. The low activity in the gastrointestinal tract might be due to an inhibitory substance as reported in West Nile virus infection in mice (10) or simply due to a dilution factor in which multiplication of AL complex was confined to the intestinal wall while titration was performed with a suspension composing intestinal contents and tissues.

DISCUSSION

From our data obtained by application of immunofluorescent staining and phase-contrast microscopy to a study of the AL complex infection in human cell cultures, we may interpret our results as indicating that, upon contact of the AL complex with human cells and shortly thereafter, a transmissible factor(s) is transferred from the AL complex to and multiplies in the neighboring cells. The evidence for multiplication was shown by a marked increase in the number of fluorescent foci of AL complex–infected cells and the rise of tissue culture units of the AL complex infectivity titers as the experimental period progressed. The AL complex antigen can be visualized distinctly by staining with specific anti-AL complex fluorescent conjugate. Localization of the antigen appears to be in the cytoplasm of infected cells first as fluorescent granules and later as homogeneous fluorescence. The AL complex infection in tissue cultures is dose dependent. When a large inoculum was used, the punctate granular fluorescence appeared as early as 4 hr after inoculation and involvement of the entire cell sheet with fluorescent bizarre ameboid cells was seen on the 3rd or 4th day. When a small inoculum was given, the early pattern of AL complex infection with granular cytoplasmic fluorescence was not apparent until the 5th or 6th day. It is important to note that during early infection, the nuclear morphology of those cells with AL complex antigen appears normal and is indistinguishable from that of the uninfected cells. This strongly suggests that AL complex infection is indeed a result of transfer of subcellular elements from cell to cell.

We do not have direct evidence that the subsequent appearance of bizarre ciliated ameboid cells with AL complex antigen is the result of multiplication of transferable elements. To have this, initiation of AL complex infection with

cell free preparations is necessary. The fact that preparations from disrupted AL complex by repeated freezing and thawing did not initiate an active infection in human cells is disappointing. The presence of cytoplasmic fluorescent specks in cells inoculated with the frozen-thawed material may simply be a result of phagocytosis or pinocytosis of disrupted AL complex cell debris. However, if the transferable factor(s) in the AL complex is a very labile agent, once it leaves the intact cell, or if the multiplication of the transferable factor(s) requires cellular factor(s), freezing and thawing may be a procedure too severe to dissociate the transferable factor(s) from the AL complex. Further investigations on these possibilities are required.

Inoculations of chick embryos with AL complex by both allantoic and amniotic routes produced deaths with antigen demonstrable in various organs. 10-day-old embryos inoculated with AL complex amniotically and incubated at 32°C showed the highest rate of infection. It has been reported (11) that the optimal temperature for "lipovirus" infection in tissue cultures and in chick embryos was 32°–34°C. By immunofluorescent staining, specific AL complex antigen was localized in the cytoplasm of groups of hepatic parenchymal cells, hemopoietic cells, pulmonary interstitial, intestinal walls, and brain cells. The bizarre ameboid cells as seen in infected tissue cultures were not encountered in tissue sections of inoculated embryos. Titration of individual organs showed significant amounts of AL complex in infected tissues. These data suggested that there was active multiplication of AL complex in inoculated chick embryos. Unfortunately, frozen sections after immunofluorescent staining were not suitable for examination with phase-contrast microscopy to depict the exact morphology of these infected cells, and examination of paraffin sections did not reveal appreciable histopathological changes. Perhaps electron microscope studies of such infected tissues might give us better information.

Preliminary work in our laboratory also showed tissue culture fluids containing AL complex can produce encephalitis in mice following intracerebral inoculations (12). AL complex antigen was seen in neurons and infectivity was recoverable from inoculated mouse brain suspensions. Since antibody against "lipovirus" has been demonstrated in a high proportion of human serum from various areas (1, 13), and the pathogenic effect of AL complex can now be demonstrated in both chick embryos and mice, further investigations to elucidate the possible effect of this agent to human infections are warranted.

SUMMARY

Tissue culture cells of human origin (HeLa, Lich, and AV_3) were inoculated with the AL complex. By immunofluorescent staining, AL complex antigen was detectable in the cytoplasm of infected cells as punctate fluorescent granules during the early stage and as homogeneous fluorescence during the late stage of infection. By combining fluorescence and phase-contrast microscopy, many

infected cells with cytoplasmic AL complex antigen were shown to have a normal nuclear morphology indistinguishable from uninfected cells. Initiation of AL complex infection was interpreted as occurring by transfer of transmissible factor(s) from cell to cell by contact and mutiplication of such factor(s) therein.

Chick embryos were susceptible to AL complex infection following allantoic or amniotic inoculations. Antigen and infectious AL complex were demonstrable in the liver, brain, intestines, lungs, and embryonic membranes. Further investigations on AL complex and its relation to human disease are suggested.

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EXPLANATION OF PLATES

PLATE 127

Fluorescence photomicrographs from Ektachrome EH135 film, daylight type.

FIG. 1. Smear of Lich seed of AL complex suspension. Note round and crenated cells with homogeneous fluorescence in cytoplasm. × 290.

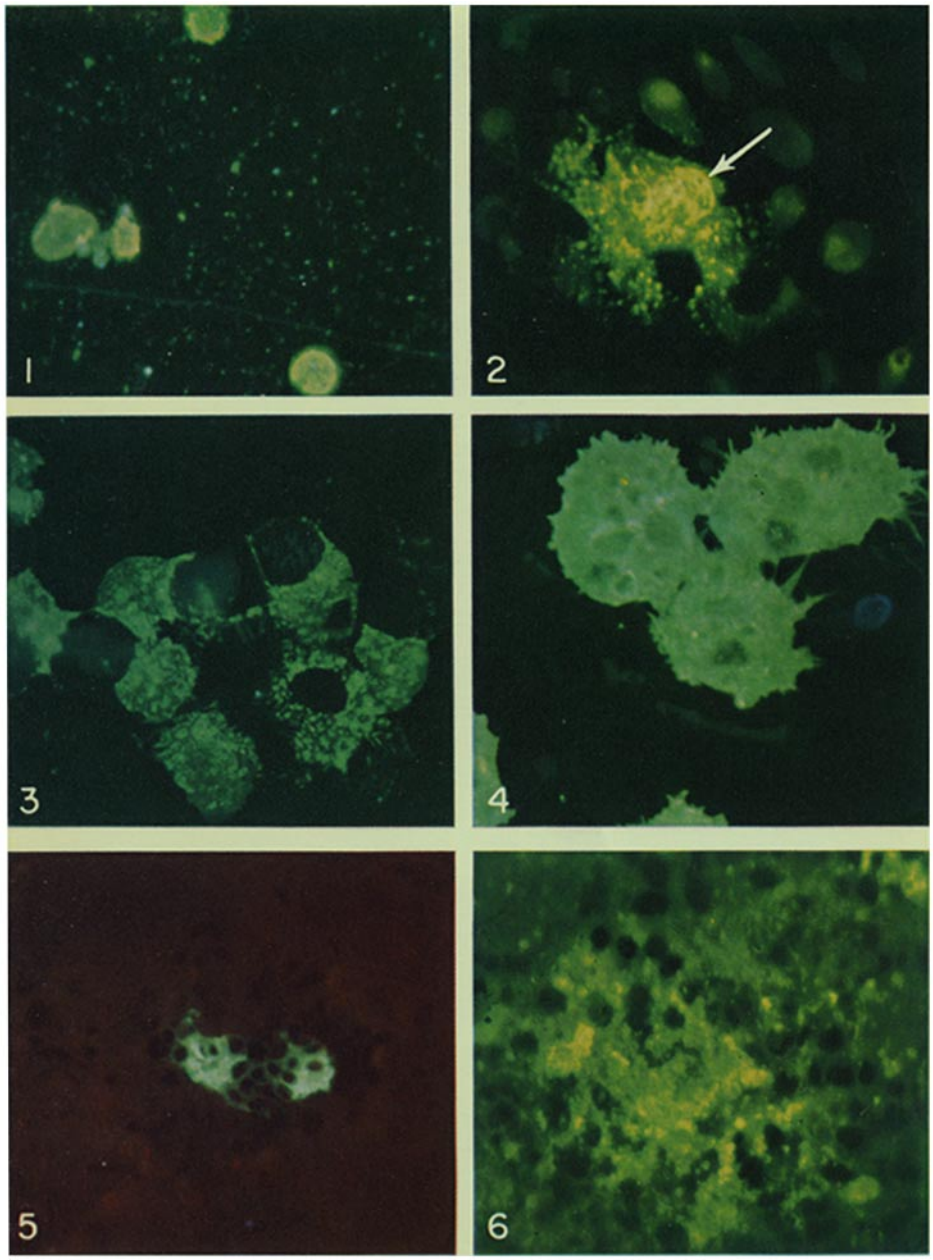
FIG. 2. Lich cells 4 hr after inoculation with AL complex. A fluorescent focus with several Lich cells having cytoplasmic granular fluorescence surrounding a central cell (arrow) with homogeneous cytoplasmic fluorescence suggesting spreading of transmissible factors to neighboring cells from the central cell. × 290.

FIG. 3. Lich cell 48 hr after inoculation with AL complex. Much AL complex antigen is seen as granular fluorescence in the cytoplasm. × 290.

FIG. 4. Lich cell 80 hr after inoculation with AL complex. Bizarre shape ameboid cells with homogeneous cytoplasmic fluorescence and ciliated protrusions. Note also multiple vacuoles in cytoplasm. × 580.

FIG. 5. Section from the liver of a chick embryo inoculated with AL complex. Homogeneous cytoplasmic fluorescence representing AL complex antigen in a group of homopoietic cells. The background has an orange color due to counterstain with rhomdamine labeled bovine albumin. × 360.

FIG. 6. Section from the liver of a chick embryo inoculated with AL complex. Granular cytoplasmic fluorescence in a group of hepatic parenchymal cells. × 580.



(Liu and Rodina: Immunofluorescent studies of AL complex)

PLATE 128

Figs. 7, 9, and 11 are photomicrographs from fluorescence microscopy and Figs. 8, 10, and 12 are from phase-contrast microscopy. Each pair represents exposure of identical microscopic fields to show that cells with AL complex antigen in the cytoplasm did have nuclear morphology indistinguishable from those neighboring cells without AL complex antigen. Pictures taken with Kodak TriX film. All magnifications × 360.

FIGS. 7 and 8. Human amnion (AV_3) cells 68 hr after inoculation with HeLa seed of AL complex.

FIGS. 9 and 10. Human amnion (AV_3) cells 66 hr after inoculation with Lich seed of AL complex.

FIGS. 11 and 12. HeLa cells 68 hr after inoculation with HeLa seed of AL complex.

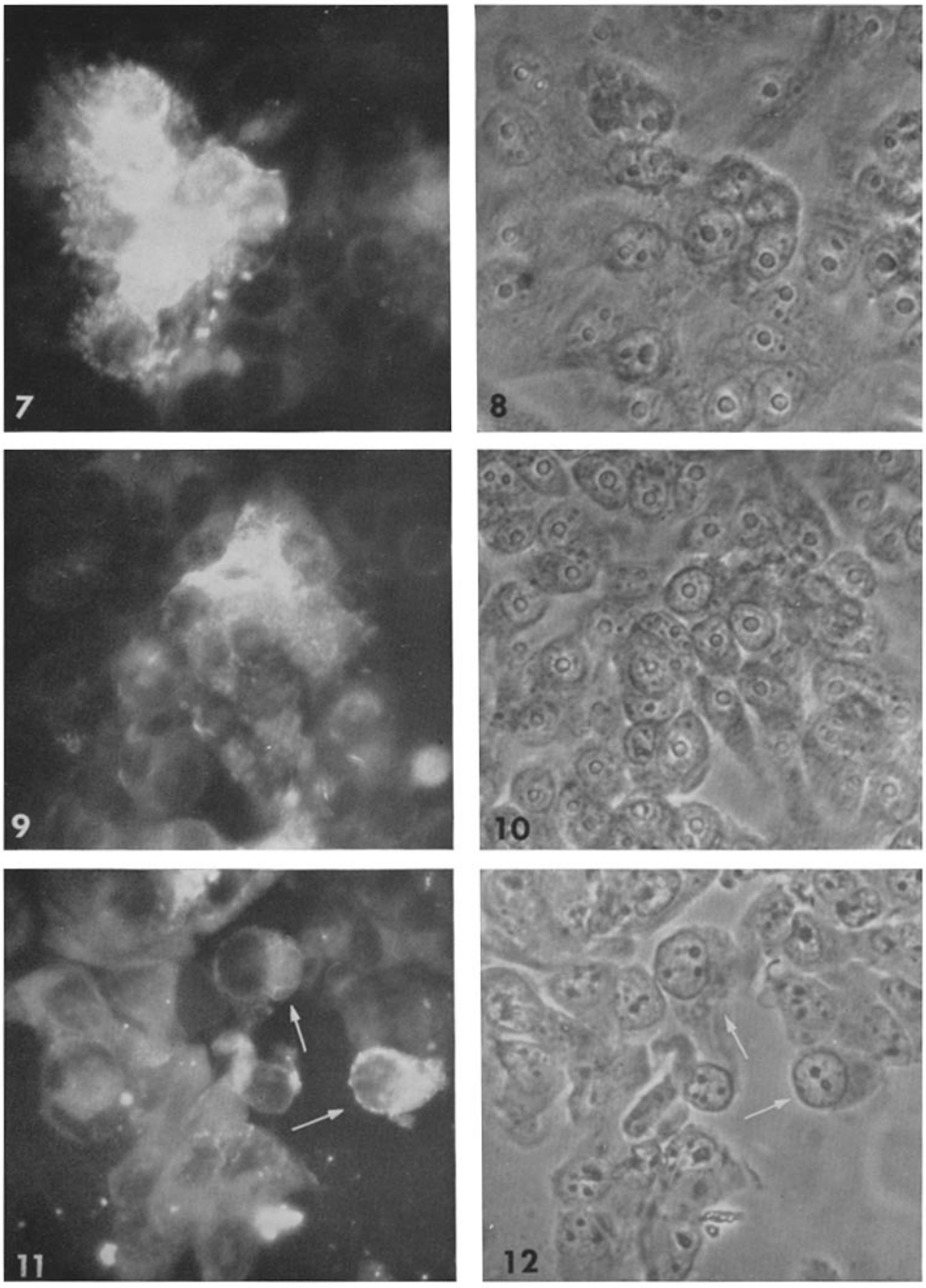


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PLATE 129

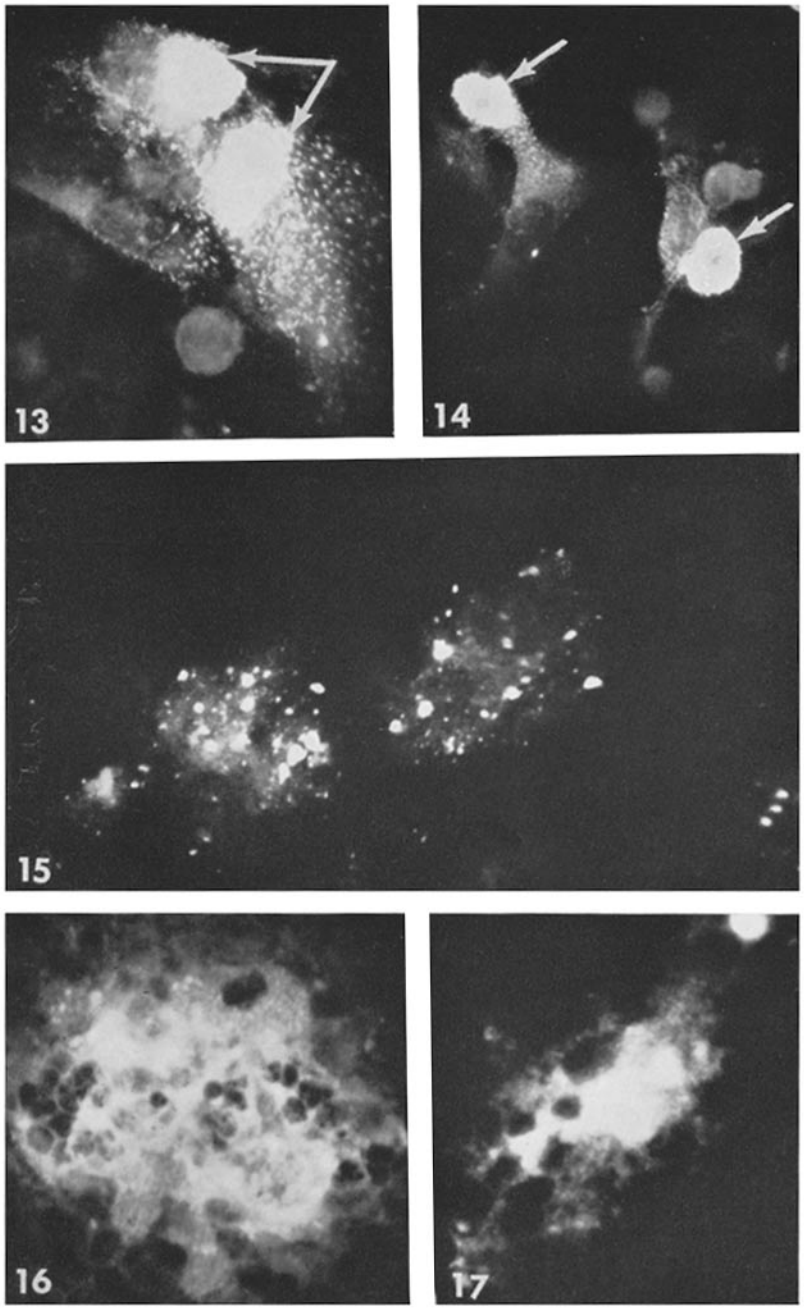
Photomicrographs of fluorescence microscopy. White areas represent AL complex antigen.

FIGS. 13 and 14. HeLa cells 140 hr after inoculation with 1000 tissue culture units of Lich seed AL complex. Note the appearance of cells with granular fluorescence contiguous to crenated round cells (arrows) with intense homogeneous cytoplasmic fluorescence. These cells have an appearance similar to Fig. 2 in which a large inoculum of 50,000 tissue culture units was used. × 360.

FIG. 15. Fluorescent specks in the cytoplasm of Lich cells inoculated with the supernate of frozen and thawed HeLa seed of AL complex. The fluorescent specks are larger and more irregular in shape than the fluorescent granules seen in tissue culture cells inoculated with unfrozen AL complex suspension. × 360.

FIG. 16. A group of cells in the intestinal wall of a chick embryo inoculated with AL complex. × 360.

FIG. 17. A group of neurons in the brain of a chick embryo inoculated with AL complex. × 360.



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