A LIPOGENIC TOXIN RELEASED THROUGH THE INTERACTION OF A NEW CYTOPATHIC AGENT (LIPOVIRUS) AND CULTURED HUMAN CELLS*

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The characterization of a transmissible agent capable of inducing DNA and thymine degradation was described in the preceding report (1). Evidence indicates that the surface coating of the agent is rich in lipids, and for this reason it is suggested that this agent be tentatively referred to as a lipovirus. This report describes the demonstration of a toxic factor found in human cell cultures infected with this lipovirus. This toxin, non-transmissible serially, is capable of inducing severe fatty degeneration of cultured human cells. Preliminary *in vivo* studies are also described.

Materials and Methods

The cultivation of primary human amnion or "liver" cells and the production of the transmissible agent were similar to that described in the preceding report (1). The lipogenic toxin was found in the supernatant fluid after the removal of infectious particles by centrifugation at 14,000 RPM for 1 hour in a Servall SS-1 angle head centrifuge; this has been described in the section on the production of the cell-free form of this transmissible agent in the preceding report (1). Unless specified otherwise, toxin prepared from liver cells nourished in Holmes' synthetic medium was used.

Induction of Fatty Degeneration.—Primary human amnion, "liver," or mouse fibroblast cells were grown as monolayers on glass coverslips about 6 to 8 mm in diameter. Each coverslip was transferred to a stoppered culture tube (15 × 150 mm) containing 0.7 ml of nutrient medium (5 per cent inactivated horse serum in Eagle's basal medium). 0.3 ml of toxin was added to each culture. The culture was placed on a roller (12 revolutions per hour) at 36°C. After 72 hours, the coverslip was removed, rinsed once in Eagle's basal medium, fixed in formalin, and stained with oil red O or Sudan IV (2). Appropriate controls were included in each experiment.

For the analysis of fatty acid, cells were grown in milk dilution bottles and were harvested by the usual trypsinization. The trypsinized cells were washed 3 times with 10 ml each of Eagle's basal medium. The washed cells were then kept frozen at below −20°C.

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Lipid Analyses.—Total fatty acids were determined by Dole's method (3). Gas chromatographic analyses were done as described elsewhere (4) using a Research Specialties Company instrument equipped with a strontium ionization detector.

DNA Determination.—The washed cells, after lipid extraction, were assayed for DNA by the diphenylamine reaction (5).

Histologic Studies.—Animals were sacrificed by etherization and tissue slices were fixed in 10 per cent neutral formalin. Frozen sections were stained with either Sudan IV or oil red O and hematoxylin. Paraffin-embedded sections were stained with hematoxylin and eosin.

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RESULTS

Histochemical Evidence of Fatty Degeneration.—2 to 4 days after the addition of toxin, fatty degeneration was regularly produced in the primary human amnion, "liver" cells, and L strain of mouse fibroblasts nourished in 5 per cent inactivated horse, human, or calf serum diluted in modified Eagle's basal medium (1). Fatty degeneration was indicated by a significant increase of sudanophilic droplets in the cytoplasm of toxin-treated cells as compared to untreated control cultures (see Fig. 1). These sudanophilic droplets were extractable with ether-alcohol. Similar fractions prepared from liver cultures killed by freeze-thawing failed to induce a similar increase of sudanophilic droplets. Like most fatty degenerative changes, this process was reversible after the removal of the toxin. Prolonged exposure, however, frequently led to slow disintegration of the affected cells. Passages of such disintegrated cultures failed to induce fatty degeneration of recipient cultures, indicating the absence of replication of the factor responsible for fatty change. There was suggestive evidence that overcrowded cultures showed less fatty changes.

Release of Toxin from Other Culture Systems Infected by the Lipovirus.—To exclude the possibility that the lipogenic toxin is released only by lipovirus-infected "liver" cultures nourished in Holmes' chemically defined medium, similar fractions obtained from lipovirus-infected "liver," chick embryonic, and primary human amnion cultures, all nourished in 5 per cent inactivated horse serum diluted in a modified Eagle's basal medium, were tested for lipogenic activity. Six such fractions have been tested; all induced extensive accumulation of sudanophilic droplets in the cytoplasm.

Preliminary Characterization of the Lipogenic Toxin.—The toxic fraction retained its lipogenic property after storage at 37°C for 10 days and 0–4°C or below −60°C for at least 1 1/2 years. Heating at 58°C for 30 minutes eliminated the lipogenic activity. Dialysis against 1000 times its volume of 0.85 per cent sodium chloride at 0–4°C for 4 hours with constant stirring failed to remove its activity. Two lots of toxin, which had been treated with 0.1 mg of crystalline trypsin (pH 8.0) at 37°C for 1 hour followed by the addition of 0.5 mg of soybean trypsin inhibitor, retained their lipogenic activity. A mixture of 0.3 ml toxin and 0.1 ml 4 per cent gamma globulin kept at 0°C for 1 hour induced

fatty changes in liver cultures similar to that induced by toxin not treated with gamma globulin. The gamma globulin used in this experiment was kindly furnished by Dr. Geoffrey Edsall of the Massachusetts Department of Public Health; it was prepared from about 1000 units of outdated human blood and antibody against poliovirus type 1 was demonstrable at 0.2 per cent. Serums collected from rabbits immunized with the lipovirus and its toxin by procedures described in the preceding report (1) did not neutralize this lipogenic activity when tested at a dilution of 1/10. Various lots of toxic fractions varied in their lipogenic potency. 1 of 8 preparations. obtained from liver cultures nourished in Holmes' medium, was found completely inactive; none was found lipogenic when tested at a dilution of 1/10. It should be emphasized that no attempt was made to detect partial reduction of lipogenic activity in these preliminary studies.

Failure to Demonstrate Similar Lipogenic Activity from Cultures Infected by Other Viruses.—Since fatty degeneration of mouse ascitic cells infected by certain arbor viruses, and HeLa Cells infected by Coxsackie B3 virus have been described (6), experiments were designed to determine whether or not a lipogenic toxin (dissociable from infectivity) can be demonstrated in cultures infected by other viruses. The following types of preparations have been tested:

1. Liver cultures in Holmes' medium were infected with vaccinia or herpes simplex viruses. After complete cell destruction, the culture fluid was centrifuged at 14,000 RPM for 1 hour in a SS-1 Servall angle head centrifuge, and the upper ⅔ of the supernatant fluid was collected. Since this fluid was still infectious, it was kept at 37°C for 7 days and was tested for lipogenic activity. At this time, fluid obtained from herpes-infected culture was no longer infectious, while that from vaccinia produced only early cytopathic change 3 days after introduction to a recipient culture. It should be noted that the lipogenic toxin of the lipovirus was stable at 37°C for 10 days.

2. Fluids from chick embryonic cultures infected with Rous sarcoma and parainfluenza 1 viruses; mouse embryonic cultures infected with polyoma virus; liver cultures infected with adeno 3, polio 1, and Coxsackie B1 viruses; conjunctival cultures infected with polio 2 virus; and allantoic fluid from chick embryos infected with influenza virus group A strain W.S. were centrifuged at 2000 RPM for 10 minutes. The supernatant fluids of Rous and polyoma virus–infected cultures were tested on liver cells. Those obtained from the remaining viruses were tested on mouse fibroblasts, strain L.

Small increases in visible fat were noted in cultures treated with polio 2, influenza A, and parainfluenza 1 viruses. These increases were slight in comparison with those induced by the lipogenic toxin. The other viruses were completely inactive.

Increase in Total Fatty Acid Content of Toxin-Treated Cells.—To find out whether the appearance of fat droplets in toxin-treated cells was due to an

alteration of the physical state of pre-existing lipids or due to an increase in uptake and/or synthesis, total fatty acids of toxin-treated and control cultures were determined. The results of 4 experiments are shown in Table I. A five- to elevenfold increase in total fatty acids per unit weight of DNA is demonstrated in 3 of the 4 experiments. Experiment 2, which shows only a slight increase, was performed during the early phase of this work. These results indicate that the lipogenic toxin induced a significant increase in the uptake of lipid from the culture medium and/or in lipid synthesis.

TABLE I

Lipogenic Toxin on the Total Fatty Acid and DNA Ratios of Cultured Human Cells

Exp.*	Cell‡	Toxin	DNA§	Total fatty acid‖	Total fatty acid/DNA
1	PA	No	0.14	1.3	9
	PA	Yes	0.05	4.7	94
2	L	No	0.26	2.5	10
	L	Yes	0.18	2.6	14
	L	Yes	0.12	2.3	19
3	PA	No	0.08	0.2	3
	PA	Yes	0.07	1.1	16
4	L	Heated toxin	0.59	3.6	6
	L	Heated toxin	0.70	3.4	5
	L	Unheated toxin	0.58	33.8	59
	L	Unheated toxin	0.58	28.7	49

* Different lots of toxin were used in each experiment.

‡ PA = primary human amnion, and L = liver.

§ Expressed as mg.

‖ Expressed as microequivalents .

Constituent Fatty Acids of Toxin-Treated and Control Liver Cells.—The relative percentage composition of the fatty acids of toxin-treated cells, control cells, and serum is given in Table II. There was an apparent increase in C_{18} dienoic acid at the expense of C_{18} monoenoic acid in the toxin-treated cells, while other fatty acids were not much affected. If liver cells behave similarly to strain L fibroblasts (4), they would synthesize little, if any, C_{18} dienoic acid, and would obtain this acid from exogenous sources such as the serum. Since there was not only an increase in the percentage of this acid in the lipids of the toxin-treated cells, but in the total fatty acid content as well (Table I), it must be concluded that at least some of the excess lipid was derived from the serum, and that the observed sudanophilia was not due simply to an alteration in the physical state of the pre-existing lipid.

Demonstration of a Cell-Clumping Factor.—Since some of the fatty acids in the toxin-treated cells apparently were derived from the serum, experiments were designed to determine whether this lipogenic toxin would also induce fatty changes in liver cells nourished in Holmes' chemically defined medium (1). It was regularly observed that 1 day after the addition of the toxic fraction most of the cells were detached from the coverslip while some remained as clumps of rounded cells, making histochemical study of fatty changes difficult. (See Fig. 2). The presence of 1 per cent human, horse, rabbit, guinea pig, or calf serum in the test system prevented cell clumping or detachment. A human gamma globulin preparation when tested at a final concentration of 0.4 per cent failed to prevent cell clumping. This cell-detaching factor differed from the

TABLE II

Major Fatty Acids of Normal and Toxin-Treated Liver Cells

Fatty acid		Relative per cent		
Chain length	Degree of saturation	Normal cells	Toxin-treated cells	Serum
14	Saturated	4.3	1.9	0.4
16	Saturated	16.7	17.5	10.6
16	Monoenoic	10.3	7.1	1.4
18	Saturated	12.1	15.6	25.1
18	Monoenoic	33.3	23.8	12.4
18	Dienoic	22.3	33.2	49.1

Column packing: 20 per cent Craig polyester succinate on chromosorb W (Wilkens Instrument and Research, Inc., Walnut Creek, California); temperature: 180°C; argon flow rate: 60 ml/min. Small amounts of other fatty acids were not included in these calculations. Values are based on the average of duplicate analyses of two different samples.

lipogenic factor in its stability to heating at 58°C for 30 minutes. It was inactivated, however, by boiling for 5 minutes. As with the lipogenic factor, the cell-detaching factor was stable at 0–4°C or below −60°C for at least several months; was non-dialyzable; was inactive when tested at a dilution of 1/10; and was not demonstrated in similar preparations obtained from cultures killed by the vaccinia and the herpes simplex virus, or by freeze-thawing. 1 of 6 preparations tested thus far was found inactive.

Preliminary Study of the In Vivo Effect of the Lipogenic Toxin.—White Swiss mice, weighing approximately 20 gm, did not show any overt effect after receiving repeated parenteral injections of the toxin. Groups of 4 mice received 5 daily injections of 0.5 cc of toxin, either intravenously or intraperitoneally, and were sacrificed serially 1 to 10 days following the last injection. The livers of the toxin-treated mice showed moderate increases in the amounts of lipid, as compared with control animals receiving comparable injections of Holmes' medium. This lipid was seen as an accumulation of sudanophilic droplets within

the cytoplasm of the liver cells centrally and midzonally. No consistent changes were found in the liver cells on hematoxylin and eosin staining. Sudan IV preparations of the other major viscera, including the central nervous system, were not remarkable. In 2 further experiments, groups of 15 white mice and 9 white rats were sacrificed 3 to 22 hours after a *single* intraperitoneal injection of 0.5 cc of toxin. Under these conditions there was no apparent difference in the amount of sudanophilic lipid in the liver cells of the test animals as compared with equal numbers of appropriate controls. The significance of the suggestive findings in the above assays, which employed repeated injections, will require testing in much larger numbers of animals.

DISCUSSION

The release of a toxic product capable of inducing fatty degeneration in cultured human and mouse cells from lipovirus-infected cultures appears well established. That this is another unique property of this lipovirus is indicated by our failure to effect the release of a similar toxin from cultures infected by the vaccinia, herpes simplex, adeno 3, polyoma, polio 1 and 2, Coxsackie B1 parainfluenza 1, influenza A, and Rous sarcoma virus. The fact that it is dissociable from infectivity distinguishes it from other viral toxins which are intimately associated with infectious particles.

This demonstration of a diffusible toxin which is capable of producing extensive fatty degeneration in cultured human cells stresses the importance of examining diffusible products of virus-infected cells for biological activities. With the exception of interferon (7) or interferon-like substance (8), published reports on biologically active diffusible products of virus-infected cells are conspicuously meager. There are several reports describing cell-detaching factors from cells infected by the adeno- or polioviruses (9); these factors are neutralized by specific antiserum and are, therefore, presumably specific viral components.

The potential importance of this lipogenic toxin can be comprehended more readily if one takes into consideration other unusual properties of the lipovirus (1). The lipovirus is presumably non-immunogenic, survives in special cells (*e.g.*, sarcoma 180 cells) *in vivo* for at least 2 months, and presumably does not circulate, owing to the presence of the ubiquitous lipoprotein-like serum inhibitor. It is tempting to speculate whether, in the appropriate *in vivo* situation, infection with or survival of the lipovirus might be accompanied by slow, continued release of the toxin into the circulatory system with resultant fatty changes at susceptible sites. To substantiate this hypothesis, one would have to establish some replication of the lipovirus in experimental animals, demonstrate its lipogenic activity *in vivo*, and achieve repeated isolations from clinical materials. Although our preliminary experimental results indicate a slight to

moderate increase in sudanophilia of the livers of white Swiss mice receiving this toxin under the described experimental conditions, further *in vivo* studies are required because of the small number of animals used. Our failure to re-isolate the lipovirus was described in the preceding report (1); the clinical materials used, however, were exclusively the acute phase blood collected from infectious hepatitis patients. Isolation attempts, using other clinical materials, are currently being made. Although chick embryos and white Swiss mice did not develop overt disease following the introduction of the lipovirus (1), it is not known whether limited multiplication of this virus occurs in the apparently healthy animals. Experiments designed to elucidate this point are now in progress.

SUMMARY

The release of a toxic product from cultured human cells infected by a new cytopathic agent (the lipovirus) was described. This toxin was dissociable from the infectious particles. It induced sudanophilia of human and mouse cells, an increase in the total fatty acid content, and a change in the major constituent fatty acids. Similar toxin was not demonstrated in cultures infected by the vaccinia herpes simplex, adeno 3, polyoma, polio 1 and 2, Coxsackie B1, para-influenza A, and Rous sarcoma viruses. Preliminary characterization indicated that this toxin was resistant to tryptic digestion and could not be dialyzed or neutralized by human gamma globulin. It was inactivated at 58°C for 30 minutes, but stable at 37°, 4°, and −60°C. The significance of these findings is discussed.

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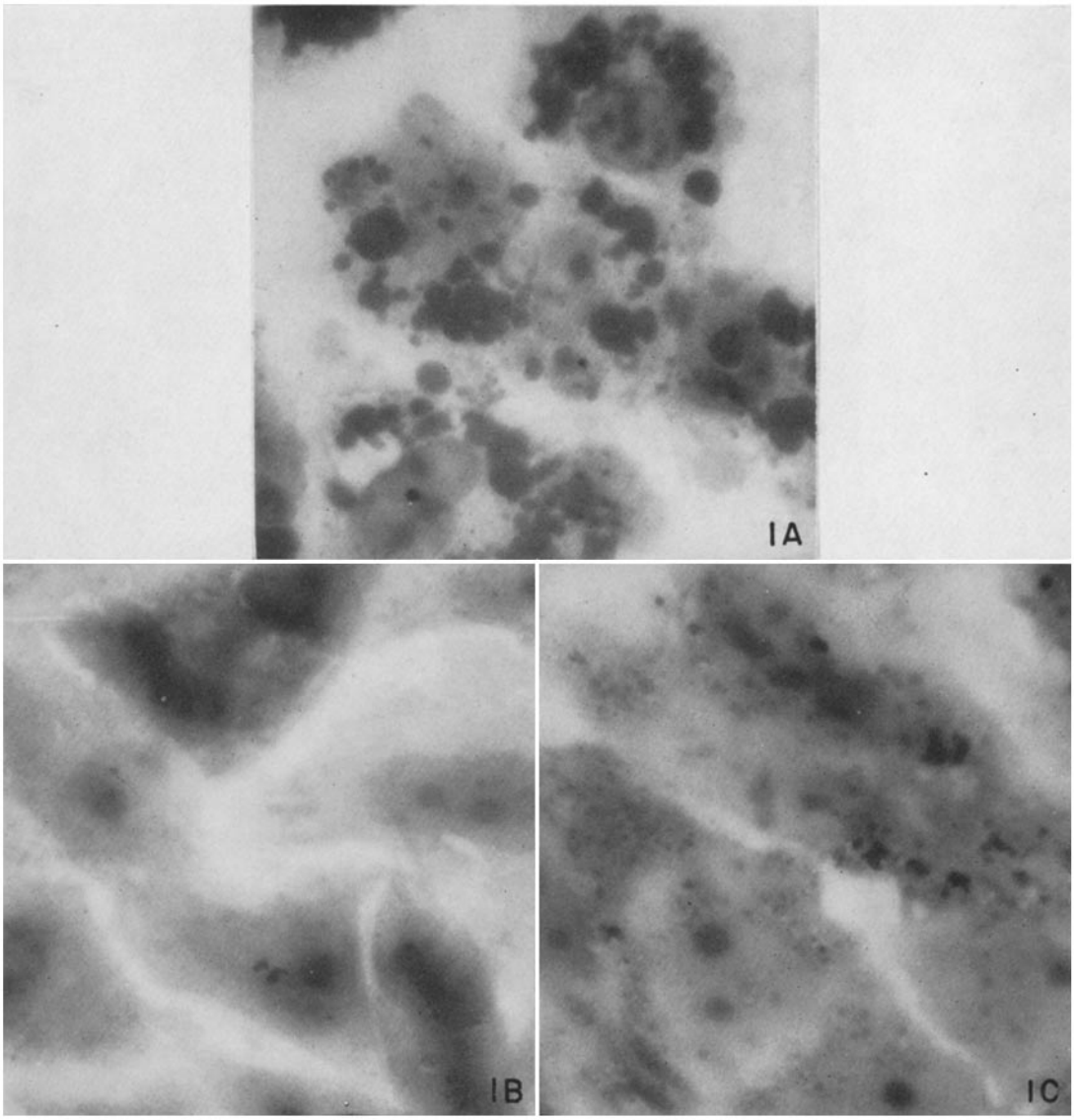
EXPLANATION OF PLATES

PLATE 104

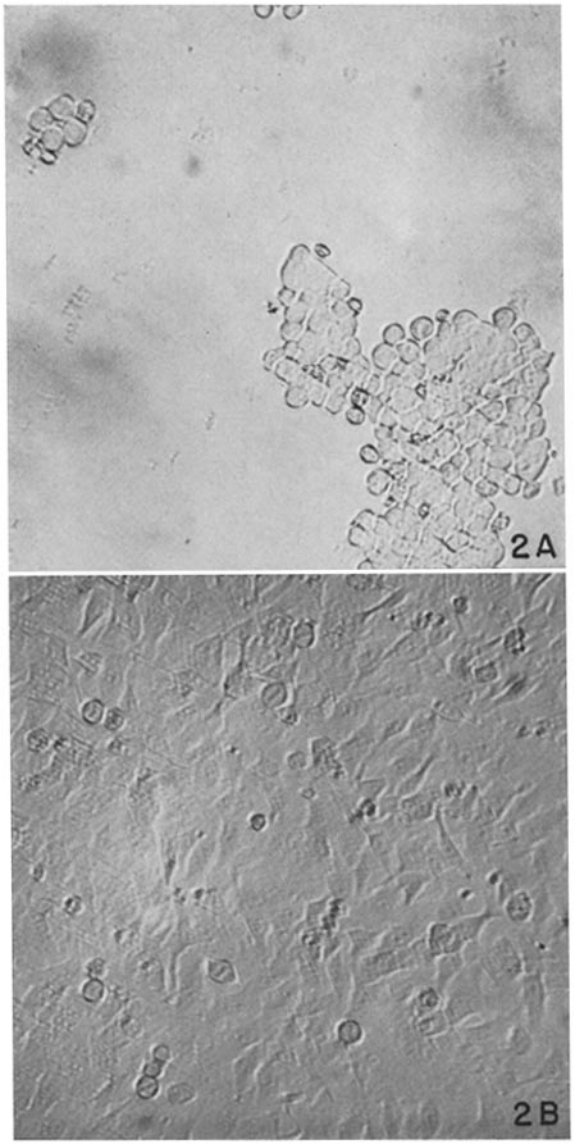
FIG. 1. Sudanophilia in cultured liver cells. *A.* toxin-treated; *B.* control; and *C.* area of maximum sudanophilia seen in some control cultures.



(Chang *et al.*: Cultured human cells)

PLATE 105

FIG. 2. "Clumping" of liver cells. *A*. treated with cell-clumping factor; and *B*. control.



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