Properties of a Transmissible Agent Capable of Inducing Marked DNA Degradation and Thymine Catabolism in a Human Cell.* (26558)

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Following the establishment of a strain of epithelial-like cells from a biopsied specimen of human liver in 1954(1), attempts were made to propagate the infectious hepatitis virus in this cell. A transmissible cytopathic agent appeared during one of those attempts. Although the origin and identity of this agent has not been ascertained, its many unusual features, its history and general properties are described in this report. The unique property of inducing marked DNA degradation and thymine catabolism in the "liver" cells is described in an accompanying report(2).

Materials and methods. Cell cultures. Monolayer roller cultures nourished in a nutrient medium consisting of 5% horse serum, preheated at 56°C for 30 minutes, in Eagle's basal medium have been used exclusively since 1956(3). Prior to 1956, a nutrient medium consisting of 20% heated horse serum in Scherer's MS(4) was used. Chick plasma, chick embryo extract and several human sera had also been used in establishment of this cell line. The "liver" cell culture(1) was found contaminated with a pleuropneumonia-like organism in 1956. Since 1957, following prolonged treatment with tetracycline, the culture has been apparently free of any such contaminants when tested on Edward's serum-agar(5), nutrient agar containing 15% horse blood and thioglycollate broth.

History of the agent. In December 1954, 4 "liver" cultures were inoculated with blood freshly drawn from 4 hepatitis patients. The blood specimen was collected on the 1st or 2nd day of jaundice during an outbreak of infectious hepatitis in a state institution for mentally defective children. About 30 days later, a blind passage was made by transferring cells and media from these 4 cultures into one single "liver" culture. In 20 more days, rapidly progressing cytopathic change appeared. This cytopathic change may be readily transmitted to other liver cultures by transferring some degenerated cells. More than 100 serial passages have been made. The data to be described are based on studies made in 1959 and 1960 on this agent which has undergone numerous passages in the "liver" culture since 1954. Unless specified otherwise, the "liver" culture was used exclusively in experiments described here.

Results. Cytopathology. Following the transfer of 0.2 ml of medium and degenerated cells from a completely degenerated culture (previously infected with the agent) to a healthy culture, degeneration usually appeared in 1 or 2 days and progressed rapidly to completion within 2 or 3 days. The degeneration is characterized by cytoplasmic granularity followed by cell rounding and shrinkage. Infected shrunken cells apparently remained intact morphologically for at least 4 weeks when kept at 36°C. Stained preparation of partially degenerated cultures showed characteristic nuclear lesion consisting of basophilic globular bodies frequently situated next to the nuclear membrane (Fig. 1). These basophilic globules are Feulgen positive, and are regularly seen in infected "liver", infected mouse fibroblasts (strain L) and in those successfully infected primary human amniotic cells.

Due to factors yet to be identified, the infecting process may at times show 3 other features: (1) marked prolongation of the incubation period; (2) establishment of a "chronic" infection, and, (3) reappearance of colonies of healthy cells in apparently completely degenerated cultures. In 2, the culture appeared partially degenerated but continued to shed infectious material for at least 2 months and was capable of converting thymidine-2-C^{14} to $C^{14}O_2$. In 3, the colonies which re-emerged were found destroyed following reintroduction of fresh agent.

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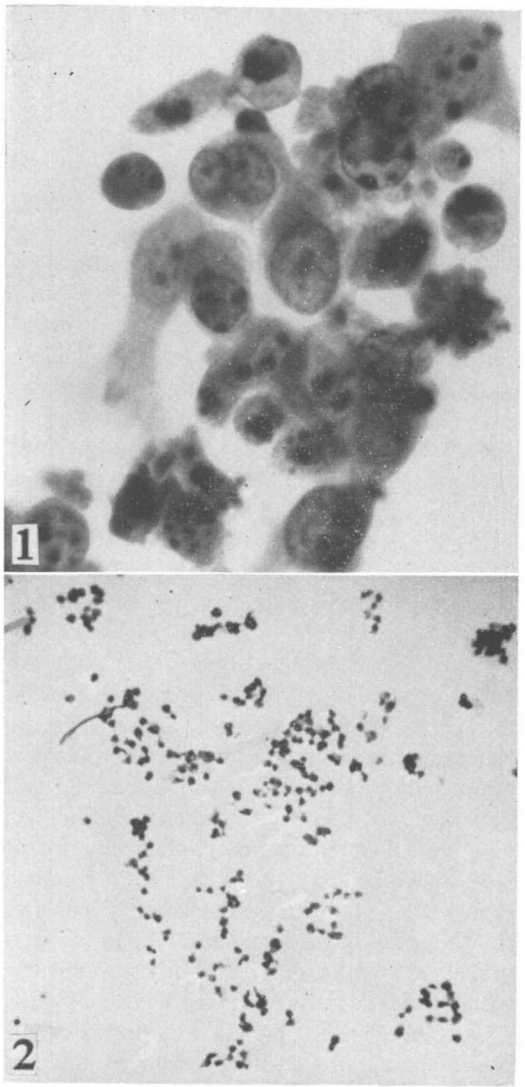

FIG. 1. Basophilic globular bodies in nuclei of infected "liver" cells.

FIG. 2. Infective portion of a degenerated "liver" culture. Note relative uniformity in size and shape of these "shrunken cells." Diameter is slightly larger than that of a human erythrocyte.

Some physico-chemical properties. The infectivity of this agent was cell associated; the fluid phase of infected culture did not contain demonstrable infectious material. The infectious component was completely sedimented by centrifugation at 2000 rpm for 10 minutes in a horizontal centrifuge (International) and was retained by a membrane filter (Millipore) with pore size of 5 μ. The material retained by this filter was infectious and consisted of many shrunken cells relatively uniform in size (about 8-12 μ in diameter) and shape (Fig. 2). The infectivity of an infected culture was destroyed by 1 cycle of freeze-thawing (occasional exceptions were encountered) but was partially retained for at least 16 weeks at 2-4°C, and 8 weeks at 36°C. Infected mouse fibroblasts cultures also retained their infectivity after heating at 56°C for 1 hour. Attempts to release infectious particles by homogenization (with a Potter homogenizer) resulted in marked or complete loss of infectivity. Exposure to .85% sodium chloride solution buffered at pH of 5 and 8 with M/15 phosphate for 24 hours at 25°C did not detectably affect its infectivity or cause the release of infectious particles. In such studies, failure to produce characteristic cytopathic changes in the "liver" cell 14 days after infection is arbitrarily considered negative for infectivity.

Susceptibility of different types of cells in vitro. Using an inoculum capable of causing complete degeneration of the "liver" cells in 3 or 4 days, cytopathic changes leading to complete destruction were produced in the following cell lines: conjunctival, HeLa, KB, FL and appendix derived from human tissues, and mouse fibroblast, strain L, and sarcoma 180 II of murine origin. Interestingly, when infected with a similar dose, primary explants of postnatal human amniotic cells (more than 10 trials) and renal cells (2 trials) or simian renal cells (3 trials) failed to show distinct cytopathic changes. With a larger infective dose, the primary amnion culture may also show characteristic degenerative changes.

Immunological study. Infected "liver" cultures, showing partial or complete degeneration, neither hemagglutinate nor hemadsorb human group "O", sheep or chick erythrocytes. Using as antigen infected mouse fibroblast L or "liver" cells in a serum free medium, negative complement fixation reactions were obtained with the following serums: (1) paired acute and convalescent serums from 5 infectious hepatitis patients (fresh blood specimens used in the original isolation were obtained from 3 of these 5 patients): (2) two convalescent varicella serums of known high antibody titers against varicella

antigen; (3) two convalescent rubella serums; (4) one adenovirus convalescent serum of high antibody titer; (5) two convalescent serums from patients with undiagnosed fever, and (6) a pool of serum from 10 healthy adults. Pooled horse serum (from 6 horses) and mouse serum (20 white Swiss mice) also gave negative CF reaction. Indirect fluorescent antibody study revealed no demonstrable fixation of antibody on the nuclear lesions of infected human "liver" or mouse fibroblasts; serums of category 1 were used. Although all the aforementioned serums also failed to neutralize this agent, these neutralization tests had to be considered inadequate since the specifically involved shrunken cells were used as the infectious material.

Other properties. This agent does not produce any visible illness in baby or adult white Swiss mice by intracerebral or intraperitoneal routes. Chick embryos survive infection by the yolk sac route, and rabbits, by intravenous or intraperitoneal routes. When infectious material was plated on blood agar or Edward's serum-agar medium and incubated in an atmosphere of 5% CO_2 in air at 36°C, no visible growth could be detected after 7 days. Similarly, no detectable growth appears in thioglycollate broth. Addition of tetracycline at 10 units per ml medium does not affect the progression of the cytopathic changes.

Failure to isolate similar agent. Several attempts to reisolate a similar agent from the 4 original specimens kept frozen at below −60°C were unsuccessful. Similarly, several other frozen acute hepatitis bloods and pooled stool specimens from 2 epidemics of infectious hepatitis failed to yield any transmissible cytopathic agent. It must be emphasized, however, that these attempts were made with frozen material and that freeze-thawing readily inactivates this agent. Isolation study using fresh acute hepatitis bloods is currently being made.

Discussion. Data obtained to date fail to establish the origin and identity of this agent. Serological findings obtained thus far neither establish nor exclude its immunological relationship with the infectious hepatitis virus. Various methods for release of this agent from the specifically involved shrunken cells are currently being investigated so that neutralization tests may be more adequately performed. Irrespective of its origin and identity, many of its characteristics are unusual for a virus infectious for human cells. These unusual features are: appearance of large basophilic Feulgen positive globular bodies in the nucleus; greater susceptibility of established cell lines as compared to the primary amnion or kidney; complete association of infectivity with the specifically involved shrunken cells; remarkable stability of infectivity of the shrunken cells and apparent rapid loss following procedures which interrupt its structural integrity, such as freeze-thawing and homogenizing, and induction of host DNA degradation and thymine catabolism, described in the accompanying report (2). Certain resemblance to the varicella-zoster group of viruses may be mentioned. The association of the infectivity with the degenerating cell and loss of infectivity following freeze-thawing and homogenizing have been reported for tissue culture propagated varicella-zoster viruses(6). Absence of eosinophilic intranuclear inclusions, failure to demonstrate viral proteins specific for varicella by the complement-fixation tests and the reaction of the cultured cells, to name only a few factors clearly establish the non-identity of the varicella-zoster group of viruses and this agent.

A logical hypothesis for the great stability of infectivity of the specifically involved shrunken cells and almost instantaneous loss of infectivity following mechanical disintegration of these cells is as follows: (1) the agent is enzyme-sensitive, and some of the cells are held at locale not accessible to the enzymes, and (2) the loss of structural integrity of the shrunken cells results in prompt enzymatic inactivation of the agent.

The rapid destructive action of this agent on the rapidly growing established human cells in contrast to its benign effect on the relatively dormant primary amniotic cells suggests a remote possibility of an oncolytic agent. Its ability to destroy the rapidly growing mouse fibroblasts, strain L, and sarcoma 180 II cells in contrast to its lack of

pathogenicity for the mouse, and its capability of inducing DNA depolymerization and thymidine catabolism in the susceptible cell are also in keeping with the requirements of an effective oncolytic agent. This possibility is being explored.

Summary. The appearance of a transmissible cytopathic agent during the first blind passage of pooled human "liver" cultures previously infected with 4 acute infectious hepatitis bloods is described. The infectivity of this agent in the "liver" culture was cell-associated. The infective degenerated "liver" cells retained their infectivity for at least 16 weeks at 4°C and 8 weeks at 36°C. Heating for 1 hour at 56°C failed to destroy completely the infectivity of infected mouse fibroblasts. One cycle of freeze-thawing or homogenizing, however, abolished the infectivity with occasional exceptions. Characteristic cytopathology consisted of cytoplasmic granularity, cell rounding followed by diminution of cell volume leading to morphologic picture of shrunken cells. During an intermediate stage of degeneration, characteristic intranuclear basophilic globules were seen regularly; the globules were Feulgen positive. The established cell line was more susceptible to the destructive action of this agent than primary cells. Preliminary immunological study failed to establish the identity of this agent.

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Appearance of Marked DNA-Degrading and Thymine Catabolic Activities in a Human Cell Infected with a Transmissible Agent.* (26559)

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The preceding paper described the history and general properties of this unidentified transmissible cytopathic agent(1). The appearance of DNA-degrading and thymine catabolic activities in infected "liver" cultures is described in this report.

Materials and method. Cell cultures. Roller cultures were used exclusively. The "liver" cells(2) were grown directly on glass in monolayer. Nutrient medium consisted of 5% horse serum, preheated at 56°C for 30 minutes, in Eagle's basal medium fortified with double strength amino acids and the non-essential amino acids at 0.2 mM(3). Certain modifications of this basal medium were also made(4).

C^{14} labelled substrates. Thymidine-2-C^{14}, uridine-2-C^{14}, and uracil-2-C^{14} with specific activity of 6.29, 0.65 and 1.1 mC per mMole respectively, purchased from New England Nuclear Corp., were used at final concentrations of 0.5 μC per ml medium.

C^{14} labelled culture. "Liver" cultures grown in media containing either thymidine-2-C^{14} or uridine-2-C^{14} for 48 to 72 hours, were rinsed 3 times with 2 ml of regular medium, without C^{14} substrate, then allowed to grow for another 24 to 48 hours in the regular media.

Fractionation of C^{14} labelled cell culture. CO_2 was first absorbed with an alkaline trap suspended in the gaseous phase of the culture

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