period. By contrast, in the experimental group there was a decrease only after 24 hours of starvation, and this change was not significant. The differences between the means for the two groups were statistically significant after 48 and 96 hours of fasting.

Previous studies (*7*, *8*) of rats subjected to prolonged starvation have shown that rapid depletion of liver glycogen takes place, reaching its lowest concentrations during the first 24 to 48 hours. This phase is followed by a gradual increase, but the liver glycogen remains far below concentrations in nonfasting animals. Muscle glycogen and plasma glucose concentrations also diminish during starvation, but the changes are less dramatic.

The liver glycogen changes in our control animals during starvation are similar to those described (*7*); however, the relative maintenance of the stores of liver glycogen in our experimental animals during starvation appears to be unique. Animals studied in the past have never exhibited comparable concentrations of liver glycogen during prolonged fasting, regardless of the sex or strain of the animals or the nature of the diet before starvation.

The amount of incorporation of undecanoate into adipose tissue triglyceride in our animals is in agreement with the mean of 31.4 percent reported by Campbell and Hashim (*9*) in weanling rats fed a diet rich in triundecanoin for 4 weeks. Calculations show that when undecanoate constitutes approximately one-third of the fatty acids of depot fat in the rat, the stores of carbohydrate precursors other than protein are increased more than threefold.

During fasting, free fatty acids, including those with odd-numbered carbon chains, are mobilized in increasing quantities from adipose tissue stores and are utilized by liver and extrahepatic tissues. The odd-carbon fatty acids are broken down in the same sequence as even-chain fatty acids, except that the final thiolytic cleavage of the C_5 fatty acid residue yields propionyl coenzyme A (CoA) rather than acetyl CoA. As shown by Ochoa and co-workers (*10*), propionyl CoA can be carboxylated, forming methylmalonyl CoA which isomerizes in two steps to form succinyl CoA. The succinyl CoA in turn is convertible to glucose and glycogen.

Since animals enriched with undecanoate appear to differ from normal animals only in respect to the substantial expansion of nonprotein glucogenic units stored in depot fat, they should prove valuable in studies of the influence of the carbohydrate stores on the physiologic response to starvation.

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3 June 1969

Human Liposarcomas: Tissue Cultures Containing Foci of Transformed Cells with Viral Particles

Abstract. *Viral particles occurring in foci of human liposarcoma cells in tissue culture are morphologically similar to the sarcoma viruses of the avian and murine species. Antibodies in the serum of the patient from whom the culture was originated reacted with cytoplasmic antigens in the original liposarcoma and in the cultured liposarcoma cells.*

Many avian sarcoma virus strains have been isolated since Rous in 1911 (*1*) described a filterable agent etiologically associated with a naturally occurring sarcoma of chickens. However, no comparable agents associated with sarcomas of any mammalian species were known until the discoveries of the murine sarcoma viruses (*2*) which are similar in many respects to their avian counterparts. Both contain RNA, are about the same size, have similar morphology, and are formed by a process of budding from the plasma membrane (*3*). Virus particles of this characteristic morphologic type (type C of Bernhard) can usually be demonstrated by electron microscopy in the sarcomas induced by these agents.

Under certain circumstances, both the avian and murine sarcoma viruses can induce sarcomas in which the intact virus particle cannot be found (*4*). Yet, the defective viral genome is present in the avian neoplastic cells because a group specific antigen of the sarcoma virus can be demonstrated in these tumor cells (*5*). Furthermore, intact infectious virus particles can be recovered upon cultivation in vitro of the sarcoma cells with normal embryo cells to which leukemia virus has been added as a helper. The leukemia virus supports the necessary synthesis of coat components of the sarcoma virus which are required for infectivity (*6*). Both the murine and avian sarcoma viruses induce foci of morphologically altered cells on infection of normal susceptible cells in tissue culture (*7*).

Since the sarcoma viruses of the avian and murine species are so similar, it might be expected that these viral characteristics would also be typical of human sarcoma viruses, if they exist. There is no direct evidence for the role of viruses in the etiology of human neoplasia. However, immunologic evidence suggests the association of an infectious agent with human osteosarcomas. Antibodies to osteosarcomas were demonstrated by immunofluorescence in the serums of patients with this disease and their close associates (*8*). Also, hamsters develop a low incidence of osteosarcomas after inoculation with extracts of human osteosarcomas (*9*).

We now describe the appearance of foci of cells in a tissue culture derived from a human liposarcoma. These foci contain abundant viral particles quite similar to the avian and murine sarcoma viruses. Antibodies in the serum of the patient from whom the culture was originated reacted with cytoplasmic

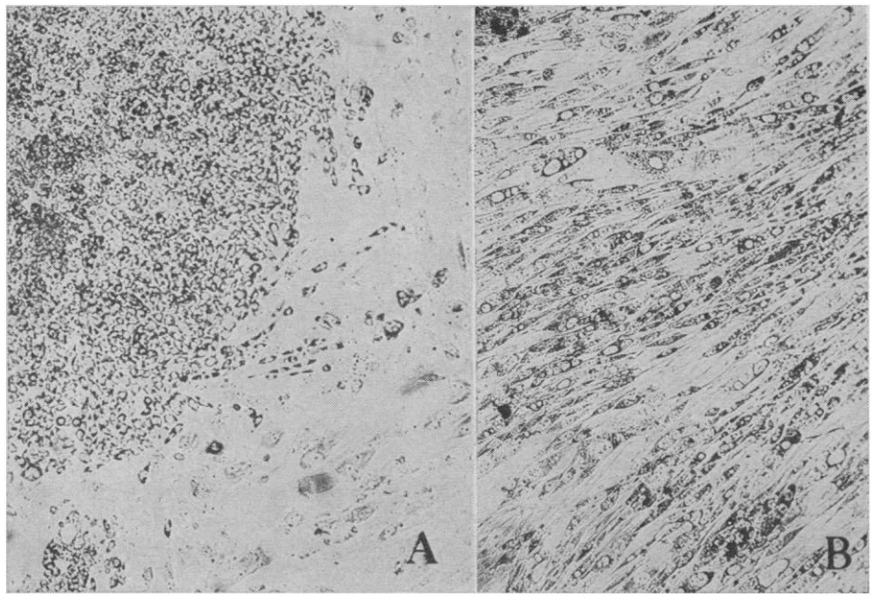

Fig. 1. (A) Focus of densely packed liposarcoma cells appearing within monolayer of sarcoma cells (× 40). This focus is the type in which virus particles were found. (B) Monolayer of liposarcoma cells obtained when culture shown in (A) was subcultured. There are numerous fat droplets in the cytoplasm (× 40).

antigens in the original liposarcoma and in the cultured liposarcoma cells, as demonstrated by immunofluorescence.

The culture was initiated from a malignant pleural effusion of a 60-year-old male with a liposarcoma originating on the right thigh. Cytological examination of the pleural fluid revealed numerous liposarcoma cells. The cells were sedimented from the pleural fluid by centrifugation (1500*g*) for 15 minutes and washed three times in Gey's balanced salt solution. The pellet was suspended in tissue culture medium RPMI (Roswell Park Memorial Institute) 1640 containing fetal calf serum (20 percent), 100 units of penicillin, and 50 μg of streptomycin per milliliter (Grand Island Biologicals). The cells were plated into Falcon T-60 plastic flasks where they slowly formed a confluent monolayer and exhibited contact inhibition. The medium was changed twice weekly; cells were subcultured every 2 weeks. The primary culture was of mixed morphology consisting of large multiform liposarcoma cells (either round or fibroblastic in shape) that contained multiple large fat droplets, large and small fibroblast-like cells without fat droplets, and an occasional round epithelial type cell (Fig. 1A). After the second subculture, the growth rate and metabolism of the cells decreased considerably, the culture just maintaining itself. Then, 30 days after the last subculture and 57 days after the culture was initiated, foci of closely packed, small, lipid-containing cells appeared in most flasks of the third and fourth passage cultures (Fig. 1A). The rapidly dividing cells in the foci lost contact inhibition and formed multiple layers at many sites in each flask. The metabolism of the culture increased, and the medium became acid more rapidly so that frequent medium changes became necessary. Several isolated foci of cells were explanted into T-30 flasks, but they did not grow well and barely maintained themselves for 4 to 6 weeks. When the mixed culture of large liposarcoma cells and foci of smaller and more rapidly dividing cells was subcultured, only the fibroblastic and fat-containing liposarcoma cells grew out. This culture now grew very slowly and took 4 to 6 weeks to become confluent in a T-60 flask which had been subcultured at a ratio of 1 : 2. However, for the next 3 months the culture maintained its original morphology as a liposarcoma without further focus formation. With light microscopy, abundant droplets of fat were seen in the cytoplasm of the liposarcoma cells (Fig. 1B); these droplets could be stained with sudan red and black.

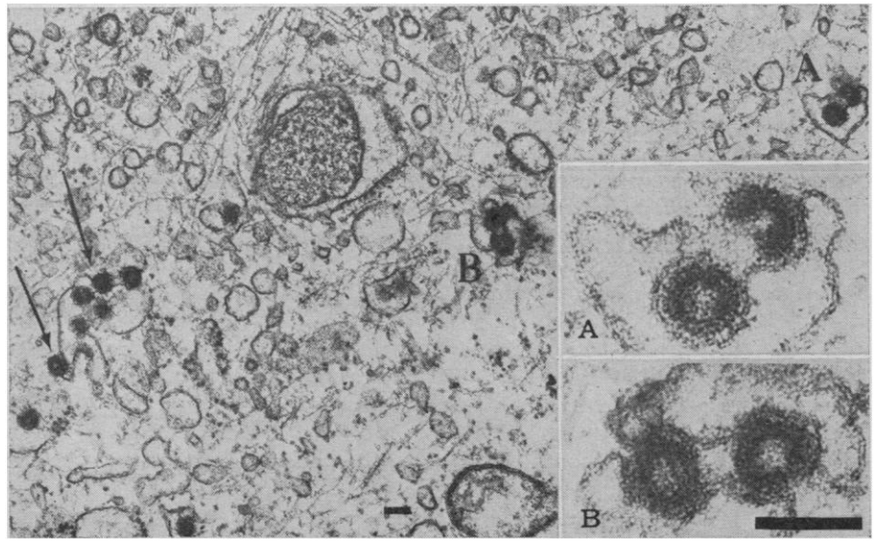

Fig. 2. Thin section through cytoplasm of a cell in a focus of liposarcoma cells. In this particular region, no lipid granules are present. Note groups of dense particles budding into intracytoplasmic vesicles. Insets (A and B) are enlargements of cytoplasmic areas *A* and *B*, respectively. Bar represents 100 nm.

Flasks containing multiple foci of liposarcoma cells from cultures of the third and fourth passage were prepared for electron microscopic examination 3 and 6 weeks after the appearance of foci in these flasks. Additional cultures were prepared for examination over the next 2 months after the culture had stopped forming foci.

For electron microscopy, cells were fixed as a monolayer for 1 hour in 1 percent chrome-osmium (*10*), rinsed in 0.5 percent aqueous uranyl acetate (*p*H 5.0) for 30 minutes, rapidly dehydrated with ethanol, and finally embedded in Epon-Araldite. Desired cellular areas were cut from the plastic sheet and mounted on plastic capsules for ultramicrotomy. Sections (1 μ thick) were stained with toluidine blue and observed by bright-field and phase optics. Ultrathin sections were

stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 1A electron microscope.

As seen with the light microscope, the major portion of the cytoplasmic volume of a typical focus of liposarcoma cells was occupied by numerous small droplets of lipid. With electron microscopy, however, one could identify the usual cell organelles in the remaining available space and, in addition, many small smooth-membraned cytoplasmic vesicles. Associated with many of these vesicles were electron-opaque particles similar to the immature C-type particles of Bernhard (*11*) (Fig. 2). As they bud into the vesicles, the particles have the characteristic three distinct layers or shells with an electron-lucent nucleoid (*12*). Virus particles have been found in all of ten different foci examined and in as many as 50 percent of the cells observed in a given focus. Although no particles were seen budding from the plasma membranes, a few mature particles have been found in the extracellular spaces. The virus particle has an outside diameter of 85 to 90 nm. The diameter limited by the second layer is approximately 65 to 70 nm, and the electron-lucent nucleoid measures 30 to 35 nm. The particles appear singly, scattered throughout the cytoplasm, but also in groups of several within a single vesicle or in the dumbbell shape reminiscent of some murine leukemia viruses (*13*). All attempts to find particles in cells growing between foci and in cultures which did not contain similar foci have been negative. Also, all efforts to detect similar particles in later tissue culture passages of this liposarcoma have been unsuccessful.

Immunofluorescent studies with the direct and indirect techniques were performed as described (*14*). Liposarcoma cells from the third passage were grown on cover slips in Leighton tubes and examined by the indirect immunofluorescent technique at the time virus particles were found in the culture by electron microscopy. The serum from the patient from whom the liposarcoma culture was obtained reacted with imprints of the original liposarcoma tumor tissue to dilutions of 1 : 100 and gave bright cytoplasmic fluorescence of the liposarcoma cells in tissue culture. Seven normal serums from blood-bank donors were tested and did not give similar fluorescence. The patient's globulins were then labeled with fluorescein thiocyanate and

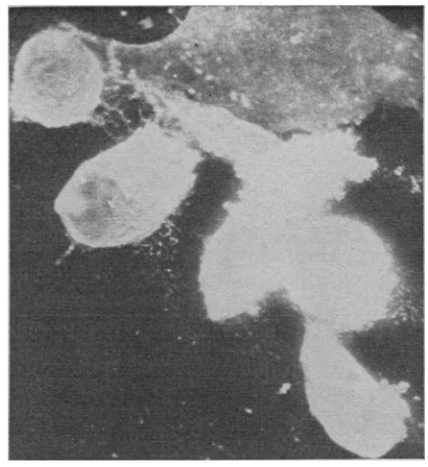

Fig. 3. Immunofluorescent photomicrograph of liposarcoma cells from culture stained with autologous globulin conjugated to fluorescein isothiocyanate. Note prominent cytoplasmic fluorescence (× 380).

tested by the direct immunofluorescent reaction against the eight subcultures of this liposarcoma. Bright cytoplasmic fluorescence was observed (Fig. 3) in up to 50 percent of the cultured liposarcoma cells. Similar fluorescence was not observed when this reagent was tested against cultures of Wi38, Hela, Wi26, or Hep 2 cells. Furthermore, the reaction against the liposarcoma cells was blocked by unlabeled serum from the liposarcoma patient.

Particles of the C type, morphologically similar to the avian and murine sarcoma viruses, have been found in foci of liposarcoma cells in tissue culture derived from a human liposarcoma. The identification and significance of these viral particles in relationship to the etiology of human liposarcomas remains to be determined. However, the following evidence suggests that the presence of these viral particles is related to the foci of liposarcoma cells appearing in these cultures.

1) Virus particles were found only in foci of a certain type of liposarcoma cells and were not found in cells of a different morphologic type between foci in the same flask.

2) No similar particles have been found on repeated examination of other cultures which no longer form this particular type of liposarcoma focus.

The virus particles observed were not due to contamination by known avian or murine viruses and cell lines because (i) no materials containing these viruses or other known viruses were being studied in the same laboratory at the time the virus particles appeared in the liposarcoma culture; and (ii) the liposarcoma cell line has a human karyotype.

It is unclear whether this culture has completely lost the viral particles or whether the remaining large lipid-containing liposarcoma cells still contain the viral genome but have lost the ability to make complete virus. In this respect, tissue cultures of human Burkitt lymphoma cells have previously been reported to transiently or permanently lose viral particles of the herpes type.

The immunofluorescent studies show that these liposarcoma cells still contain the same tumor antigen as they did when the virus particles were first found. Whether the effective antigen is a new antigen coded for by the viral genome or an unrelated tumor antigen is unknown. The effective antigen is not the complete virus itself since up to 50 percent of the cultured cells still react by immunofluorescence when no virus particles can be seen by electron microscopy. However, the immunofluorescence studies could be detecting antigens of a defective human sarcoma virus comparable to the antigens of defective avian sarcoma viruses previously demonstrated in "nonproducing" Rous sarcoma cells by Kelloff and Vogt (*6*). Attempts to rescue the potentially defective viral genome from this cell line have been unsuccessful thus far.

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Hepatitis in Marmosets: Induction of Disease with Coded Specimens from a Human Volunteer Study

Abstract. *Marmosets inoculated with plasma from three early acute hepatitis patients developed hepatitis 30 to 40 days later. Other groups of marmosets receiving preinfection plasmas from the same patients showed no evidence of hepatitis in this experiment. It is, therefore, most probable that hepatitis in marmosets represented transmission of human disease rather than activation of latent "marmoset hepatitis."*

Previous reports from our laboratory (*1*, *2*) have characterized the hepatitis occurring in two species of marmosets after the inoculation of serum or plasma obtained from patients early in the course of acute viral hepatitis. Subsequently, other investigators have suggested that this phenomenon represents the activation of latent "marmoset hepatitis" rather than the transmission of human disease (*3*). In order to help resolve this issue an experiment was designed in which preinoculation and acute-phase plasmas were obtained from three of ten human volunteers, who had been inoculated orally with infectious plasma containing the Willowbrook MS-1 strain of hepatitis virus (*4*) and who developed clinical disease 27, 29, and 31 days, respectively, after inoculation. Hepatitis was confirmed by liver biopsy. The acute-phase specimens used in our study were samples of plasma drawn on the 29th day from two of the volunteers and on the 30th day from the third (*5*). No Australia antigen (*6*) was detected by complement-fixation tests in the preinoculation, the day-33, and the day-100 (after inoculation) serums of the three human volunteers.

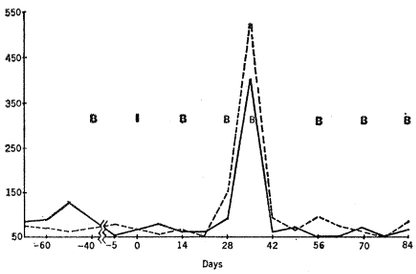

Fig. 1. Changes in serum enzyme activities of a marmoset inoculated with acute-phase plasma from patient G.C. (*B*) Liver biopsy; (*I*) inoculation on day 0. Values on the ordinate are levels of serum glutamic oxalacetic transaminase (SGOT) activity (solid line) and of serum isocitric dehydrogenase (SICD) activity divided by 10 (broken line). Methods used for these tests have been previously described (*1*). Upper limits of normal in our laboratory for marmosets are SGOT of 200 and SICD of 2000.

To insure objectivity in the interpretation of results the six specimens were coded (by Col. Marcel E. Conrad, Walter Reed Army Institute for Research) before being sent to us, and the marmoset liver biopsies were studied by one of us (L.W.) under code. Each of the unknown specimens was diluted 1 : 2 in Hanks balanced salt solution and each marmoset received 0.5 ml of a diluted specimen intravenously. Inoculated animals and their uninoculated controls were bled once weekly for the determination of serum activity of glutamic oxalacetic transaminase and isocitric dehydrogenase, and percutaneous needle liver biopsies were done at least every 2 weeks in all experimental subjects.

The results of the experiment are detailed in Table 1. Only marmosets inoculated with acute-phase human plasma developed hepatitis confirmed both biochemically and by liver biopsy. Incubation periods were fairly consistent at 30 to 40 days from inoculation to detection of first abnormal biochemical tests. The course of the disease was brief, as exemplified in Fig. 1. A single animal in the group receiving preinoculation plasma from patient G.C. had a rise of serum enzyme activities 60 days after inoculation, but these values had returned to normal 4 days later. A liver biopsy done on the 60th day showed no evidence of hepatitis.

From the results of this experiment it would seem likely that human hepatitis can be transmitted with regularity to marmosets. These specimens were obtained quite early in the disease (just before the first abnormal hepatic tests in one of the three volunteers) and for this reason they were ideal for attempted transmission. If we add to these six unknowns two other coded specimens (one proven positive and the other proven negative in human volunteers) the correct identification of which was reported in an earlier publication (*1*), we have a total of eight unknowns. Considering the marmosets as eight groups, the statistical likelihood of correct identification by chance alone is 0.37 percent. If the marmosets are considered as individuals, this chance becomes far smaller.

The suggestion that the disease observed in the experimental animals is latent "marmoset hepatitis" stems from three points. One is that the agent producing disease in marmosets has different properties (such as heat and ether stability) than those commonly attributed to human hepatitis virus. It should be remembered that the physicochemical studies of human hepatitis virus were done in various experiments with human volunteers, by using different original inocula from experiment to experiment and with a small number of total studies. A careful search of the literature shows, for example, that the dictum of ether stability of infectious

Table 1. Results of inoculation of coded specimens from volunteer patients into marmosets. Incubation period is defined as interval from inoculation to detection of first abnormal hepatic tests.

Patient	Plasma specimen	Marmosets inoculated	Marmosets showing hepatitis	Incubation days
R.F.	Preinoculation	6	0	
R.F.	Acute phase (day 29)	6	5	34, 41, 41, 41, 48
F.K.	Preinoculation	6	0	
F.K.	Acute phase (day 30)	6	5	33, 33, 40, 40, 40
G.C.	Preinoculation	5	0*	
G.C.	Acute phase (day 29)	6	5	36, 36, 36, 36, 51
	None (controls)	8	0	

* A single animal in this group had elevated serum enzyme activities on day 60 but a normal liver biopsy.