Globin was prepared by adding the hemoglobin (10 to 30 mg/ml) by drops to a solution of 0.006*M* HCl in acetone at −20°C while stirring vigorously (*5*). The total volume of acetone was 10 to 20 times that of the hemoglobin (10 to 30 mg/ml) by drops to a solution of 0.006*M* HCl acid acetone, and dissolved in 11.7 percent formic acid. The α- and β-chains were then separated by the column chromatographic method of Chernoff (*6*). When samples contained both hemoglobins A and F, the γ-chain was eluted directly before the β-chain and could be separated adequately from the other peptide chains. The identity and purity of the peptide chains were verified by peptide mapping of tryptic digests (*7*). The separated α- and β-chains were dialyzed against distilled water to remove excess urea and then were lyophilized, and the protein content was determined by the Folin-Lowry method (*8*). Portions were plated on 2.5-cm stainless steel planchets, and radioactivity was assayed in a low-background gas flow beta-counter.

Patients J.L. and N.L. were siblings with thalassemia major; they had approximately 35 and 45 percent hemoglobin F, respectively, and 3.3 percent hemoglobin A2. Their mother's hemoglobin was 3 percent F and 4 percent A2, but the family was not otherwise studied. A thalassemic patient from a different family, P.T., had approximately 10 percent hemoglobin F.

In the patients with thalassemia (Table 1) the specific activity of the β-chain was approximately one-half that of the α-chain in the hemoglobin of two members of one family, regardless of the method of hemoglobin purification, and one-seventh that of the α-chain in one member of another family. This is in sharp contrast to patients in the remission phase of acute lymphocytic leukemia or patients who had sickle cell anemia, where in both instances the specific activity of the β-chain was always higher than or equal to that of the α-chain. Since the proportion of amino acid residues in the α-chain to those in β-chain are 12 : 18 for valine and 17 : 19 for leucine, the lower β-chain specific activity found in the patients with thalassemia is even more striking.

It remains to be determined whether this unusual labeling is specific for some forms of thalassemia as compared with other diseases with deficient hemoglobin synthesis. Conceivably, this pattern of isotopic labeling could be a manifestation of excess α-chain production by cells, a slow rate of β-chain assembly, or a loss of β-chain label. This latter could theoretically occur by dilution of labeled β-chain material with nonradioactive material prior to incorporation into hemoglobin.

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Human Wart Virus: In vitro Cultivation

Abstract. *Inoculation of cell cultures of fetal skin of human and murine origin with virus extracted from human wart tissue resulted in the appearance of intracellular wart virus specific antigen, as demonstrated by fluorescent antibody techniques. Appearance of antigen was accompanied by cytopathogenicity and the accumulation of large numbers of characteristic virus particles.*

The viral etiology of human warts has been documented (*1*). The agent, 45 to 55 mμ in diameter, has been described as a DNA virus with 42 capsomeres in icosahedral symmetry (*2*). Because of its similarity to the rabbit papilloma, polyoma, and SV-40 viruses, the wart virus has been placed in the papova group (*3*).

The advantages of using a model human tumor (benign) virus system have attracted considerable attention to the human wart virus (*4*). However, there has been no unequivocal demonstration of cultivation of this agent in vivo or in vitro, other than in man (*5*). We now have presumptive evidence of the cultivation of the human wart virus in cell cultures of embryonic human and mouse skin.

Human warts (*Verruca vulgaris*) were removed surgically, immediately frozen in liquid nitrogen, and stored in sealed ampules at −70°C. Warts (at least six in each group) were pooled and pulverized in the frozen state. The resulting fine powder was suspended (100 mg/ml) in Hanks balanced salt solution (BSS) and homogenized in a glass homogenizer at 0°C. After centrifugation at 5000*g* for 10 minutes at 4°C the supernatant was put aside and the pellet was suspended to the original volume in BSS. The suspension was treated with high-frequency sound in a Raytheon oscillator (10 kcy/sec) at full output for 8 minutes. It was then centrifuged, and the supernatants of this and the previous centrifugation were pooled. Portions of these preparations were routinely examined for wart virus by electron microscopy; negatively stained (*6*) with phosphotungstic acid (PTA) they showed abundant characteristic human wart virus particles.

Antiserums were prepared in the rabbit against wart virus partially purified by differential centrifugation. The globulin fraction of pooled serums was obtained by precipitation with ammonium sulfate.

Almeida, Cinader, and Howatson (*7*) suggested that the specificity of immune globulins could be demonstrated by the formation of virus-antibody complexes as seen by electron microscopy. Accordingly, a portion of the immune globulin was mixed with an equal volume of the crude wart virus extract which was prepared from a wart pool separate from that used to immunize rabbits. The mixture was incubated at 37°C for 2 hours and then at 4°C overnight. After centrifugation at 1500*g*, the pellet was rinsed twice and suspended in phosphate-buffered saline (PBS). The preparation was stained with PTA and examined in the electron microscope. The complex resulting from the mixture of virus and immune globulin (Fig. 1) suggested the presence of wart virus specific antibody; this complex was not formed with normal rabbit serum globulin. The immune globulin was conjugated with fluorescein isothiocyanate (*8*), and the unconjugated dye was removed by gel-filtration on Sephadex G-25 (*9*).

Skin aseptically removed from a 4-month human fetus was washed, and a cell suspension was prepared by treatment with trypsin. Primary cultures were incubated for 6 days in Eagle's medium with 10 percent calf serum. Secondary cultures were prepared on coverslips in

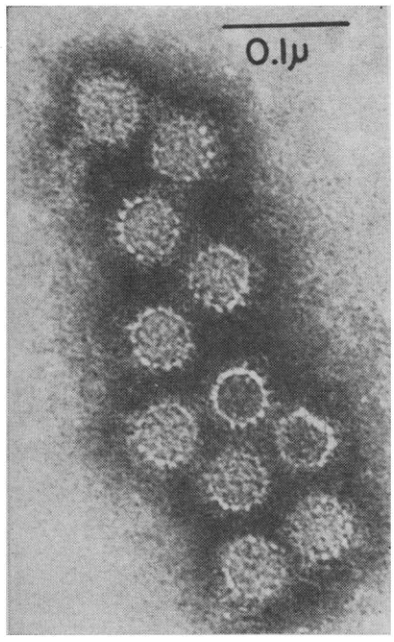

Fig 1. Wart virus in the presence of antibody. Virus particles are surrounded by a halo of antibody and clumped. A chain of five particles linked by antibody in zig-zag formation can be seen; stained with PTA.

Leighton tubes (2.5×10^5 cells per milliliter per tube). Twenty-four hours after seeding, secondary cultures were rinsed with BSS and inoculated with 0.4 ml of wart extract which had been passed through a Millipore filter. After a 3-hour adsorption period fresh medium was added, and incubation was continued at 37°C. Control cultures received either BSS or heated (100°C, 10 minutes) wart extract. Within 24 hours severe cytotoxicity was observed in cultures inoculated with the wart virus preparations; less severe cytotoxicity was also observed in cultures exposed to heated wart virus preparations. At various intervals after inoculation, coverslip cultures were removed, and the preparations were washed 3 times in PBS and fixed in acetone. The fixed preparations were first stained with rhodamine (*10*) for 30 minutes at 37°C to suppress nonspecific fluorescence. After further washing with PBS (three times) the cells were stained with the fluorescein-conjugated immune globulin for 40 minutes at 37°C. The fluorescent antibody had been previously adsorbed (*11*) with powdered mouse liver and a powder prepared (with acetone) from the human fetal skin from which the cell cultures had originated.

The coverslips were washed thoroughly with PBS, dried in air, and mounted in 25 percent glycerol in PBS. The cells were examined with a Zeiss photomicroscope equipped for fluorescent microscopy.

In cultures that had been inoculated with wart virus an intense yellow-green nuclear fluorescence appeared by the 8th day. The cells in 12-day cultures showed both marked nuclear and increasing cytoplasmic fluorescence. Parallel cultures which had been inoculated with BSS or boiled wart virus exhibited only the orange fluorescence of the rhodamine counterstain.

The cytotoxicity first observed shortly after inoculation persisted for 6 to 8 days. For this reason the appearance of nuclear fluorescence first seen in virus-inoculated cultures at 8 days may have occurred before this time only to be obscured by the cytotoxicity.

The appearance of wart virus antigen in the nucleus of inoculated cultures was accompanied by accumulation of large numbers of morphologically characteristic virus particles in the culture media. These were detected by electron microscope examination of PTA-stained preparations prepared by high-speed centrifugation (40,000*g* for 90 minutes).

The appearance of intracellular viral antigen and the accumulation of large numbers of characteristic virus particles in cultures of human embryonic skin cells suggested the proliferation of a virus morphologically and immunologically identical with the human wart virus. Similar experiments were also performed with 3rd and 6th passage human skin cells.

Because of the relative availability of materials, concurrent experiments were carried out with secondary skin cell cultures prepared from 15-day mouse (Swiss, HaICR) embryos. Secondary coverslip (22 by 22 mm) cultures in 30-mm petri plates (2.5×10^5 cells per plate) were inoculated with 0.3 ml of a wart virus preparation. After a 3-hour adsorption period, cultures were rigorously rinsed with warm culture media to remove unadsorbed virus; input virus in the final rinse could not be detected by electron microscopy. Fresh medium was added to the coverslip cultures, and incubation at 37°C in an atmosphere of 5 percent CO_2 was continued. Daily thereafter the supernatant medium along with any detached cells present was removed, and the cells were disrupted by freeze-thawing (three times) and treatment with high-frequency sound for 3 minutes. After centrifugation (1500*g*, 10 min) the supernatant was removed and centrifuged at 40,000*g* for 90 minutes. The resultant pellet was resuspended in a small volume of saline and stained with PTA for electron microscopy. Replicate cultures removed at the same intervals were prepared for fluorescent antibody study (*12*). A third set of replicate cultures was examined for cytopathogenicity by phase-contrast microscopy.

Cultures inoculated with wart virus were first positive for characteristic nuclear fluorescence on the 2nd day after inoculation; only a few cells then contained detectable viral antigen. The incidence and intensity of nuclear fluorescence increased with time. A typical 6-day preparation is shown in Fig. 2. As in the case of the human skin cell cultures, intensity of nuclear staining was diminished in later cultures showing cytoplasmic fluorescence. Viral antigen could not be detected in cul-

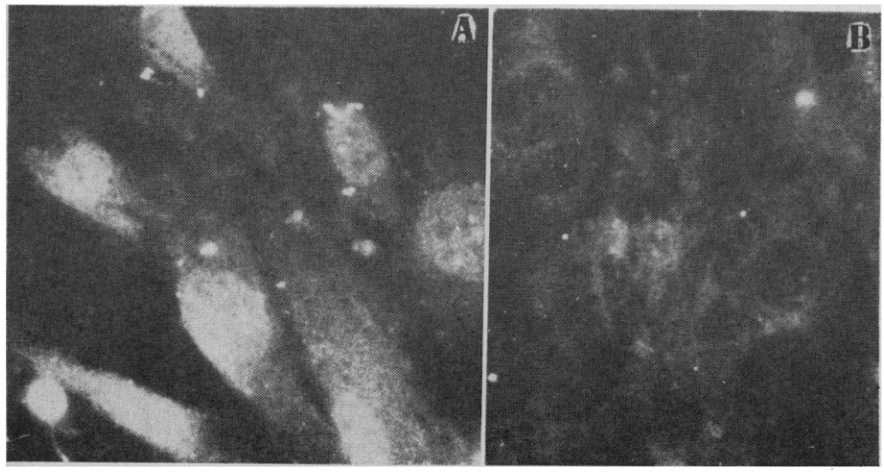

Fig. 2. Cells of mouse embryo skin stained with fluorescein-conjugated globulin prepared from antiserum to wart virus (× 800). *A*, Cells infected with human wart virus, 6 days after inoculation; note strong nuclear fluorescence. *B*, Uninfected control cells; no fluorescence was observed.

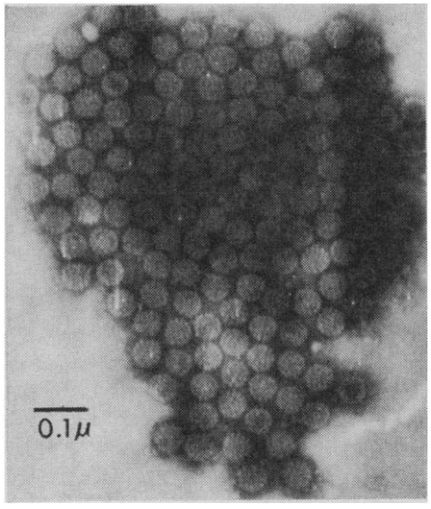

Fig. 3. Human wart virus isolated from 13-day cultures of cells of mouse embryo skin; stained with PTA.

tures inoculated with saline or boiled virus.

In mouse cell experiments, the initial cytotoxicity was less severe than that observed in experiments with human skin cultures. The mouse cells were fully recovered from the initial cytotoxicity 4 or 5 days after inoculation. At this time, cells inoculated with wart virus exhibited a cytopathogenicity (CPE) characterized by a rounding of cells and the formation of aggregates which detached from the monolayer. In continuously cultured cells the CPE, but not the initial cytotoxicity, appeared in cultures which had been inoculated with supernatant medium from infected cultures rather than with primary wart extract preparations.

The appearance of viral antigen and CPE in cultures of mouse embryo skin cells inoculated with human wart virus was accompanied by accumulation in the culture medium of large numbers of characteristic virus particles. Virus was not detected until the 4th day after inoculation. A typical "sheet" of virus particles from a 13-day culture may be seen in Fig. 3.

The wart virus used for injection and antibody production was prepared from separate pools of human warts. The cell cultures used were early passages of primary human and mouse tissue obtained from several separate sources. It therefore seemed improbable that we were dealing with a morphologically similar papova (contaminant) virus. We nevertheless used several fluorescein-conjugated antiserums prepared against other members of the papova virus group. By the aforementioned procedures, coverslip cultures of mouse embryo skin cells inoculated with wart virus were stained with antiserums prepared against human wart virus (rabbit), polyoma virus (rabbit), SV-40 virus (rabbit), and adeno-12 virus (mouse) (*13*). Only the cells stained with antibody to human wart virus showed fluorescence.

In earlier, unsuccessful, attempts to propagate the human wart virus, in which other methods were used to extract the virus, the early cytotoxicity was not observed. We have found this cytotoxicity to be dependent on *p*H. The relation between this early cell damage and sensitivity to subsequent infection warrants further study in both human and mouse cell cultures.

We have presented data from studies with fluorescent antibody and the electron microscope as evidence of the propagation in cell culture of a virus morphologically and immunologically similar to that isolated from human warts. Since we have not determined the ability of this propagated virus to produce warts in man, we have only presumptive evidence of the cultivation of the etiological agent of the human wart.

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13. Fluorescein isothiocyanate-conjugated antiserums (monospecific) against polyoma, SV-40, and adeno-12 viruses were supplied by Dr. Jeana D. Levinthal, Department of Bacteriology, Harvard Medical School.
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5-Bromodeoxyuridine: Effect on Myogenesis in vitro

Abstract. *Presumptive myoblasts, obtained by treating muscle from 11-day chick embryos with trypsin, multiply in vitro. On the 4th or 5th day in culture they abruptly fuse, form long multinucleated myotubes, and begin to synthesize myosin. Cultured cells exposed to 5-bromodeoxyuridine incorporate this analog of thymidine into their DNA. Cells with such falsified DNA are reversibly inhibited from forming myotubes and synthesizing myosin; such cells, however, continue to synthesize the various species of molecules required for cell multiplication.*

5-Bromodeoxyuridine (BUDR) is a thymidine analog incorporated into only the DNA of proliferating cells (*1*).

Our experiments were designed to determine whether or not embryonic cells with such "altered" DNA undergo normal differentiation. Rapidly proliferating presumptive myoblasts were grown in vitro in the presence of BUDR. Before and after exposure to the analog, the cells were inspected for capacity to divide, to fuse and form multinucleated myotubes, and to synthesize myosin and assemble cross-striated myofibrils.

Suspensions of mononucleated, presumptive myoblasts were obtained by digesting breast muscle of 11-day chick embryos with trypsin (*2*). The cells were plated in Leighton tubes, containing coverslips coated with chicken plasma clots, at concentrations of 3×10^5 cells per milliliter. The