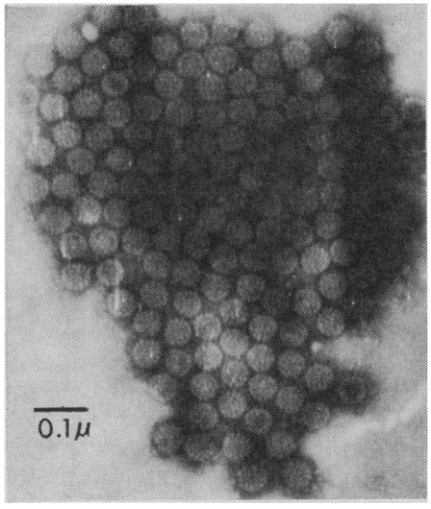

Fig. 3. Human wart virus isolated from 13-day cultures of cells of mouse embryo skin; stained with PTA.

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.
14. We thank Marie Wong, L. Johns, Jr., and P. Meyers for assistance; J. Gershon-Cohen for support and encouragement; and J. Weiner, R. Schwartz, and H. A. Marbach for supplying biological materials. Supported by a grant from the Mark Borish Chapter NFER, USPHS grant CA-06711-02, a research contract (PH 43-65-46) from the National Cancer Institute, and USPHS career award 1-K3-CA-21773-01 to M.A.R.

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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

cultures were grown in medium consisting of 11-day embryo extract, balanced salt solution (Simm's), and horse serum (1:2:2).

To detect DNA synthesis radioautographically, tritiated thymidine (*3*) was used in concentrations of 0.5 mc/ml of nutrient medium. Radioautographs were prepared as previously described (*2*). Determinations of DNA were based on the method of Burton (*4*). 5-Bromodeoxyuridine (*5*) was used in concentrations of 25 to 250 μg/ml. Incorporation of BUDR into the DNA of cultured cells was followed by cesium chloride density-gradient centrifugation (*6*). Myosin synthesis was studied with fluorescein-labeled antibody prepared against chicken myosin (*7*).

Multinucleated skeletal muscle forms by the fusion of many mononucleated cells. Once a cell fuses with another, the nuclei within the resulting myotube cease to synthesize DNA (*2*, *8*). When cultures of mononucleated muscle precursor cells that have been treated with trypsin are plated in Leighton tubes they increase in number for the first 3 or 4 days, with relatively little fusion between cells. There is a precipitous formation of long, multinucleated myotubes between the 4th and 5th days of culture. By the 8th day of culture, many myotubes are longer than 1 mm and over 20 μ in diameter, and contain several hundred nuclei. Cross-striated myofibrils which react with fluorescein-labeled antimyosin are detectable in some myotubes in 6-day cultures and are conspicuous in most myotubes after 8 days of culture. Many thousands of nuclei within myotubes have been observed, but we have never detected one in mitosis; nor do such nuclei incorporate H^3-thymidine (see, however, *9*).

The rate at which dissociated cells synthesize DNA was determined by adding H^3-thymidine to the cultures; on one or another of the first 5 days of incubation the cells were exposed for 24 hours to labeled thymidine. After a 24-hour exposure on either the 1st, 2nd, 3rd, 4th, or 5th day, the cells were immediately killed and prepared for radioautography; Fig. 1 summarizes the results. In another series the cells were treated as before but, instead of being killed after exposure to H^3-thymidine, they were washed three times and then incubated in cold medium (supplemented with 0.25 mg of unlabeled thymidine per milliliter) until the cultures were 6 days old; the results of these experiments are shown in Fig. 2. In another series the H^3-thymidine was added daily for the first 3 days of culture and then the cells were killed; between 95 and 97 percent of all nuclei were labeled. Taken together, these three experiments indicate that most cells divide at least once, and many divide three or four times, before being swept into myotubes. Thus there is ample opportunity prior to fusion for incorporation of BUDR in at least one strand; in many instances, in the two DNA strands of each double helix.

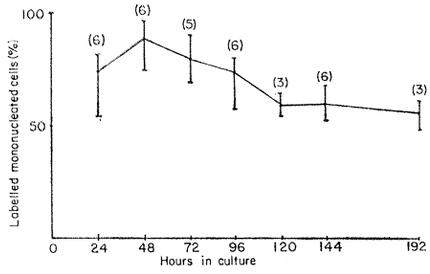

Fig. 1. The percentage of mononucleated cells which incorporate H^3-thymidine over a 24-hour culture period. The average and range are indicated; the numbers of separate experiments are shown in parentheses. Random fields of 1000 cells were counted for each experiment.

If cells are incubated in BUDR medium for the first 2 days of culture and are then placed in normal medium until the 8th culture day, typical myotubes develop. Similarly, if BUDR is present from the 4th through the 8th culture day there is no obvious difference between treated and untreated cultures. However, if BUDR is present during the first 5 days of culture, after which the cells are cultured an additional 3 days in normal medium, only an occasional short and attenuated myotube forms. For every 200 to 300 myotubes in the controls, there is approximately one myotube in the BUDR-treated series. There are fewer myofibrils, and fewer nuclei to each myotube, in the rare myotubes in the BUDR-treated cultures. Myotubes in the treated series are rarely wider than 5 μ, and are generally only a few hundred microns long. On the other hand, in BUDR-treated cultures, the cytology of the few myofibrils that do form, and the reaction of the protein in their A-bands with the antimyosin serums, are indistinguishable from the controls.

To determine whether the inhibition by BUDR is due to some unknown action on cell membranes or cytoplasm, the following experiments were performed. (i) Bromouracil, thymidine, and cytidine are pyrimidines structurally related to BUDR; their addition to the medium in concentrations ten times that of the BUDR did not interfere with myogenesis. (ii) By increasing the concentration of the original inoculum, myotubes can be made to form within 24 hours. Under these conditions myotubes form in the absence of cell multiplication (*10*) and, hence, before many cells can incorporate BUDR into their DNA. Accordingly, cultures were prepared with 5×10^6 cells per milliliter. When ten times the concentration of BUDR (0.25 mg/ml) used in the low-density cultures was added to the high-density cultures, it did not inhibit fusion or the ensuing myogenesis. (iii) If the inhibition of myogenesis is due to the competitive incorporation of BUDR into the DNA of presumptive myoblasts, such cells should be protected by the addition of an excess of thymidine to the BUDR medium. Cultures in BUDR medium to which thymidine was added in threefold (equimolar) concentrations were indistinguishable from controls.

Actinomycin, fluorouracil, mitomycin, and colchicine interfere with myogenesis (*10*) but, as they kill cells, block mitosis, or cause fission of the formed myotubes, it is difficult to interpret such experiments. It is therefore important to show that the effect of BUDR is reversible and is not due to selective killing or blocking of divisions of competent cells.

To demonstrate that BUDR did not inhibit DNA synthesis, parallel cultures with and without the analog were prepared. The DNA was measured in two BUDR-treated and two untreated (control) cultures at the end of days 1, 2, 3, 4, 5, 6, and 9. The relative amounts of DNA in treated cultures, expressed as percentages of the content of control cultures, were as follows: 106 percent, day 1; 92 percent, day 2; 98 percent, day 3; 110 percent, day 5; 125 percent, day 6; 150 percent, day 9.

The greater quantities of DNA in the BUDR series, particularly in the older cultures, is due in part to the presence of myotubes in the control cultures. Once the cell enters a myotube its nucleus is withdrawn from the mitotic population. Clearly the effect of BUDR is not due to its lethal action on multiplying cells. BUDR may re-

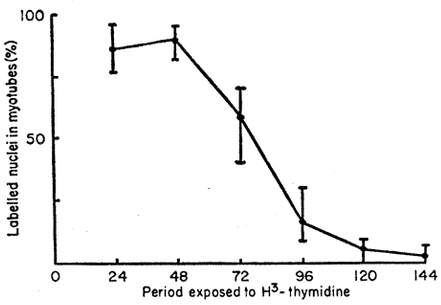

Fig. 2. Cells were treated with labeled thymidine for 24 hours on either the 1st, 2nd, 3rd, 4th, or 5th day of culture. The cells were washed and reincubated in cold medium. All were killed on the 6th day of culture and prepared for radioautography. The percentage of labeled nuclei in myotubes was determined. Each point is the average of three experiments. In each experiment 1000 myotube nuclei were counted.

duce the viability or fragment the chromosomes of some types of cells, whereas it has no demonstrable effect on other types of cells (*11*). The absence of deleterious effects of BUDR in our cultures may be due to the embryo extract in the medium; omission of embryo extract results in reduced survival and decreased mitotic rates.

That the BUDR is not selectively killing presumptive myoblasts was further demonstrated by preparing "second-generation" cultures. Control cultures and cultures treated for 5 days with BUDR were treated with trypsin and replated in normal medium for another 8 days. Second-generation cultures prepared from BUDR-treated cells formed myotubes with the same high frequency as second generation controls. These results suggest that the blockage of myogenesis depends on the incorporation of BUDR into the DNA of the involved cells. Furthermore, if BUDR-inhibited cells are permitted to synthesize new, and presumably normal, DNA their progeny again exhibit the capacity to differentiate into muscle.

The incorporation of BUDR into DNA was demonstrated by gradient centrifugation. Six million cells treated with BUDR for 5 days, and a similar number of control cells, were lysed in 1 ml of distilled water to which cesium chloride was added to produce a 7.7 molal solution. These samples were centrifuged for 24 hours at 44,770 rev/min in a Beckman model E ultracentrifuge. The DNA from untreated cultures had a density of 1.697; DNA from treated cultures, 1.733, the latter band corresponding to double strand replacement. The difference in density was equivalent to a 55 to 60 percent replacement of available thymidine positions, based on the assumption that the volume of the DNA molecule was not changed by substituting Br for the methyl group on thymine and that the DNA was composed of equal amounts of the four bases. After 3 days in culture three bands were detected by ultracentrifugation; presumably the middle band reflects replacement of thymidine by BUDR in a single strand. In other experiments cells were treated with BUDR for 5 days, washed, and then cultured for another 5 days in normal medium. The density of the DNA returned to normal. These findings confirm the biological findings, namely that the progeny of cells which had BUDR in their DNA have normal DNA when they synthesize it in medium lacking the analog.

These experiments are compatible with the possibility that, when half the thymidine positions of the DNA of presumptive myoblasts are occupied by BUDR, some functions fail to emerge (*12*) while others persist. Fusion, synthesis of myosin, and formation of myofibrils are strikingly inhibited, but the cells synthesize DNA and all those specialized molecules required for cell multiplication. It is not clear why certain synthetic functions should be inhibited by altering the structure of the DNA whereas other functions, thought to be under the same kind of genetic control, are not affected. The molecules which regulate cell division are as complex and as dependent on the proper base sequences in the DNA as are those required for cell differentiation (*13*). If the presence of BUDR interferes with proper coding of those genes required for myogenesis, the question arises as to why the BUDR does not interfere with genes regulating the events of cell division.

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Enolase: Multiple Molecular Forms in Fish Muscle

Abstract. *Starch-gel electrophoresis shows three distinct molecular forms of enolase in each of eight different species of Salmonidae. The three enolases do not appear to be artifacts of isolation, and their electrophoretic patterns are completely reproducible. The patterns are also highly characteristic for each individual species of fish, and together with the overall myogen patterns they represent unequivocal means of taxonomical identification.*

Molecular heterogeneity of specific enzymes isolated from a given source is well known (*1*). We have recently obtained evidence for multiple forms of enolase in the muscle of several species of Salmonidae. Although the cellular origin and the physical and chemical characteristics of the different forms of enolase have not yet been determined, our data show that there are three enolases in each of the species investigated. The species investigated were rainbow trout (*Salmo gairdnerii gairdnerii*), cutthroat trout (*Salmo clarkii*), eastern brook trout (*Salvelinus fontinalis*); and sockeye