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Viral Probes of Aging in the Human Diploid Cell Strain WI-38[1] (38110)

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Normal human and animal diploid fibroblast cell strains have a limited capacity to replicate *in vitro* (1–4). These cell strains, when derived from human fetal tissue, have a population-doubling potential of 50 ± 10 (1, 2). Cell strains derived from older donors reveal a population-doubling potential inversely related to the age of the donor (2, 5). It is also now becoming apparent that the numbers of population doublings to be expected from these cell strains might be related to the mean lifespan of the species from which such cells have been derived (6).

After the initiation of normal diploid fibroblast populations *in vitro* (Phase I) a logarithmic increase in cell numbers occurs (Phase II), followed by a cessation of cell division and ultimate death of the cells (Phase III) (1, 2). The Phase III phenomenon is now generally regarded to represent biological aging at the cellular level, and the nature of the phenomenon is under active investigation (3, 4). Of the several theories offered to explain biological aging and the Phase III phenomenon, the notion of Orgel has elicited significant interest (4, 7, 8). This hypothesis suggests that initial translational mistakes lead to an ever-increasing accumulation of errors in proteins, ultimately resulting in functional and structural decrements leading to manifestations of aging and subsequent cell death.

One of several experimental approaches leading to a direct test of this hypothesis is to utilize viral probes as a means of determining whether errors in protein synthesis occur at greater rates in older cells. Since viral replication depends upon the utilization of a significant proportion of host cell synthetic machinery, it is hypothesized that any potential for error production in an old cell would be amplified and more easily detected in viral progeny that replicate in such cells. If accumulation of errors in the form of abnormal proteins eventually does cause or contribute to senescence of human and animal diploid cell strains *in vitro*, this should be reflected or amplified in numbers of anomalies of virus replication and maturation in host cells approaching, or in, Phase III. This reasoning is the basis on which the following experiments were designed to test the error catastrophe hypothesis of Orgel (7, 8).

We have used an RNA virus, poliovirus type 1 and a DNA virus, herpesvirus type 1, to test the fidelity of viral protein synthesis, defective assembly of constituent viral components, and possible alterations in patterns of cytopathology as a function of *in vitro* age of normal human diploid cells. Four virus properties were assessed: (1) amount of virus produced, (2) pattern of cytopathology, (3) plaque morphology, and (4) analysis of herpesvirus proteins by polyacrylamide gel electrophoresis.

Materials and Methods. Cells. The normal human fetal diploid strain used was WI-38 (2) cultured in a single lot of Eagle's Basal Medium Diploid (BME) (9), obtained from Grand Island Biological Co., Lot D117. The BME was supplemented with a single lot of 10% calf serum (Pacific Biological Co., Lot 933C), 28 mM HEPES buffer, and 50 μg/ml aureomycin. Glass 32-oz bottles (Owens) were used for routine cultivation. Further details

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for cultivating normal human diploid cell strains have been described previously (1, 2).

WI-38 was subcultured at a 1:4 split ratio twice weekly until the number of population doublings had reached 50; thereafter, and during Phase III, cells were subcultured at a 1:2 split ratio every 3 days until cultures failed to reach confluency.

For virus production the calf serum component of the BME was replaced by 1% fetal calf serum, with 50 ml of this medium used per 32-oz bottle of WI-38 cells. Parallel cultures not infected with virus were trypsinized and cell yield per bottle determined by use of a Coulter Counter (Model Z_{B1}), in order to relate virus yield to cell numbers. The virus suspensions and cell cultures were routinely monitored for mycoplasma contamination by a method described by us previously (10) and by measuring uridine/uracil ratios (11). All tests were negative.

Viruses. Cells were infected with viruses at a multiplicity of 0.1 PFU/cell. Poliovirus type I (CHAT strain) and herpesvirus type I were kindly supplied by Dr. S. Plotkin (Wistar Institute, Philadelphia, PA). Seed virus and virus harvests were stored at −70°. Infectivity titrations were performed in microtrays (Falcon Micro Test II 3040). Each titration consisted of six replicates and the $TCID_{50}$/ml was calculated by the method of Reed and Muench (12).

Plaquing was done in 50-mm Lux plastic petri dishes. The virus was adsorbed to the cell sheet for 1 hr followed by introduction of a 1% agar overlay. After incubation at 37° for 72 hr, a second overlay containing neutral red was added, and the plates incubated at 37° for a further 18 hr. Plaque purification of the poliovirus strain was carried out by a modification of the method described by Dulbecco (13). Plaque purification of the herpesvirus was not undertaken since, as judged by plaque morphology, the virus pool was homogeneous.

In comparing virus produced from "young" and "senescent" WI-38 cells the virus from a common seed stock was passed three times at a multiplicity of 0.1 PFU/cell in cells younger than 25 population doublings, and in cells older than 50 population doublings which were undergoing no mitotic activity and were visibly in Phase III (1, 2).

Concentration of herpesvirus proteins for acrylamide gel electrophoresis. When infected cell sheets showed complete cytopathology due to herpesvirus replication, the cultures were shaken vigorously to remove remaining attached cells. The cell suspensions were frozen and thawed three times and clarified by centrifugation at 400 g for 10 min in a PR2 International Centrifuge. Supernatants were collected and again centrifuged at 400 g for 10 min. The herpesvirus was then pelleted from the supernatant at 22,000 rpm (56,500*g* max) in a 30 rotor in a Beckman Model L ultracentrifuge for 80 min. Virus pellets were suspended in phosphate-buffered saline (13), and layered on 10–50% sucrose gradients in 3½ × 1 in. cellulose nitrate tubes. The gradients were centrifuged at 25,000 rpm (113,-000*g* max) for 1 hr in a SW 27 rotor in a Beckman Model L2-65B centrifuge. Absorbance at 260-nm wavelength was monitored using a UVicord apparatus, and the virus band collected.

Purified virus was simultaneously dialyzed against 0.01 *M* phosphate buffer and concentrated to 0.25 ml, in a -p ProDiCon apparatus (Bio-Molecular Dynamics), and virus protein was estimated according to the Lowry method (14).

Acrylamide gel electrophoresis. Analysis of viral proteins by acrylamide gel electrophoresis was determined in 7.5% disc gels with an acrylamide:bis acrylamide ratio of 38:1. To 100-λ samples of solution contining 50–100 μg of protein was added 10 λ of 10% SDS in 10% mercaptoethanol, 20 λ of glycerol and 2 λ bromphenol blue solution. The solutions were boiled for 2 min, the denatured and solubilized protein layered on 8-cm disc gels, and run onto gels at a constant current of 3 mA per gel. The current was then increased to 8 mA per gel for approximately 4 hr until the bromphenol blue tracking dye had migrated to within 2 cm of the end of the gel.

Gels were removed and simultaneously fixed and stained overnight in a solution containing 0.025% Coomassie blue, 25% isopropanol, and 10% acetic acid. They were then transferred to a solution containing 0.0025% Coomassie blue, 10% isopropanol, and 10% acetic acid for 6 hr to destain, and stored in 10% acetic acid.

The absorbance of the protein bands in the

TABLE I. Number of Infectious Virions of Poliovirus and Herpesvirus Produced as a Function of Age of WI-38.

WI-38 population-doubling level[a]	Virus	Average number of cells ($\times 10^6$)	Titer $TCID_{50}$/ml[b]	Average number of infectious virions produced per cell
18	Polio I	17	$10^{6.9}$	24
20	Polio I	17	$10^{6.7}$	15
53	Polio I	7	$10^{6.3}$	14
54	Polio I	7	$10^{6.3}$	14
20	Herpes I	17	$10^{7.7}$	150
22	Herpes I	15	$10^{7.6}$	130
58	Herpes I	8	$10^{7.8}$	380
60	Herpes I	6	$10^{7.5}$	250

[a] Replicate cultures of senescent cells failed to subculture past population-doubling 54 for poliovirus and population-doubling 60 for herpesvirus. The senescent cells used were from two separate reconstituted frozen ampoules.

[b] Mean of three titrations with six replicates per titration.

gels was measured at a wavelength of 660 nm with a Gilford gel scanner attachment and Gilford recording spectrophotometer.

Results. Cytopathology. Infected cultures showed similar cytopathic effects for both viruses replicating in Phase II and Phase III cells. The first appearance of a cytopathic effect and the rate of progression was comparable in all experiments when 0.1 PFU of virus per cell was used as the inoculum.

Table I shows the average number of infectious virus particles produced per cell for both poliovirus type I and herpesvirus type I, and indicates that the Phase III cell populations support the production of a similar number of infectious virions per cell as do Phase II cell populations.

Plaque morphology. Herpesvirus type I produced plaques in WI-38 cells with shapes following the polar direction of the fibroblasts, and hence were not circular. More than 500 plaques were examined in Phase II cells, and 400 in Phase III cells. The virus plaque morphology was homogeneous in all experiments.

Table II compares the ratio of large and small plaque types for poliovirus I. Both plaque-purified and nonpurified virus was used, and as these produced identical plaque-size ratios, the combined results are presented. The arbitrarily determined plaque-size ratios found were similar for Phase II and Phase III cells.

Herpesvirus protein analysis. A typical result of an analysis of herpesvirus type I proteins by polyacrylamide gel electrophoresis is illustrated in Fig. 1, and shows that identical absorbance profiles are obtained from herpesvirus type I produced in Phase II and Phase

TABLE II. Analysis of Poliovirus Plaque Sizes Produced in Young and Old WI-38.

Population doubling level[a]	Number of plaques examined	Size	Number	Ratio of large to small plaques
20	1306	≥ 3 mm	899	2.3:1
		< 3 mm	407	
54[b]	789	≥ 3 mm	550	2.3:1
		< 3 mm	239	

[a] Virus passed three times in WI-38 at this population-doubling level before assay in WI-38 at population-doubling level 18–22.

[b] Phase III cells (replicate control cultures failed to reach confluency upon subculture).

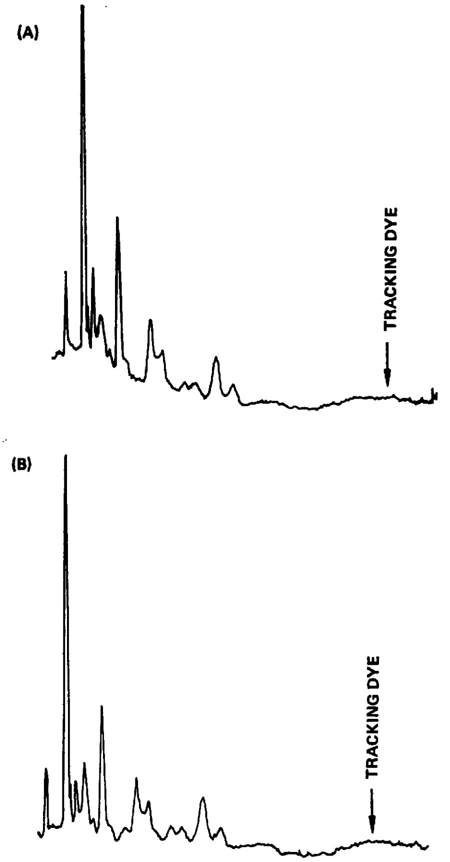

FIG. 1. Absorbance profiles of Coomassie brilliant blue-stained herpesvirus type I proteins. The virions were produced in Phase II (A) and Phase III cells (B).

III cells. The gels which produced this profile are shown in Fig. 2.

Discussion. Table I shows that the number of infectious virions produced per cell was similar, despite replication in Phase II or Phase III WI-38. Thus, even though Phase III WI-38 cells could no longer replicate, and on attempted subcultures were unable to attach to the glass substrate, they were still able to support the assembly and production of mature infectious virions to the same degree as actively dividing cells in Phase II.

As each culture was infected with approximately 0.1 PFU per cell, the rate of appearance, type, and progression of the cytopathic effect could be compared. No differences were observed on any occasion for either virus, which would suggest that no delay in virus assembly occurred in Phase III cells. This finding was further supported in comparative plaque experiments, where plaque morphology and size were also similar after passage of the virus three times in Phase II and Phase III cells, from a common virus seed stock. No morphological plaque variants were seen in either Phase II or Phase III cells with herpesvirus type I and the ratio of plaque sizes in poliovirus-infected cells remained constant after replication in Phase II and Phase III cells.

Figures 1 and 2 show that no difference was detected when the proteins of herpesvirus type I virions replicating in Phase II or Phase III WI-38 were analyzed by polyacrylamide gel electrophoresis. The absorption profile obtained was similar to that reported by Spear and Roizman (15) for highly purified virus, except that no low molecular-weight material was found in the region of the bromphenol blue tracking dye. However, when the material which had pelleted through the sucrose gradients was dialyzed and subjected to polyacrylamide gel electrophoresis a prominent peak corresponding to that shown by Spear and Roizman was seen at the position of the tracking dye. Though the pellet was shown to contain infectious virions, the lack of the small molecular-weight component in the purified virus may be related to the method of dialysis and concentration. To our knowledge, this is the first report where the -p ProDiCon has been used to simultaneously dialyze and concentrate virus particles.

During the preparation of this manuscript, Holland *et al.* (16) reported results of similar experiments that are essentially in agreement with ours. In addition to herpesvirus type I and poliovirus type I they used vesicular stomatitis virus. They showed that Phase II and Phase III cells were equally susceptible to all three viruses, that the virus produced in such cells had the same specific activity, and that senescent cells did not produce a large number of defective virions. They further showed that viruses produced in Phase II and Phase III cells have similar thermal stabilities and that mutation rates were also similar.

Our results and those of Holland *et al.* (16), therefore, do not support the concept of an error catastrophe occurring in old cells due to generalized translational errors. It is possible, however, as suggested by them (16), that errors may be restricted to specific host cell codons which do not affect virus replication.

As pointed out by Holland *et al.* (16), poliovirus induces a replicase in infected cells (17) and if the replicases synthesized in Phase III cells contain a substantial number of abnormal molecules, mutation rates should be affected. Our experimental evidence does not support this expectation.

It is also possible that an accumulation of error-containing proteins in aging cells does occur, but since viral proteins are synthesized *de novo,* the presence of such proteins would go undetected. Holliday and Tarrant (18) and Lewis and Tarrant (19) discussed this possibility when they described a lower specific activity and a higher temperature lability of enzymes found in Phase III human diploid cells as compared with those in Phase II. Although the experiments described here by no means disprove the error hypothesis, our results and those of Holland *et al.* (16) do not support it. As discussed previously (1–3, 6), we favor an explanation for the Phase III phenomenon, and for biological aging generally, based upon a specific genetic program where, as in normal biological development, gene expression and gene regulation govern the sequential production of information containing molecules. The expression of biological aging may depend upon these same fundamental mechanisms and not upon protein infidelity as the essential cause.

Summary. Poliovirus type I and herpesvirus type I were used as probes to determine

FIG. 2. Photograph of 7.5% polyacrylamide disc gels stained with Coomassie blue after electrophoresis of the denatured proteins of purified herpesvirus type I. The gel on the left is derived from virions produced in Phase II cells and that on the right from virions produced in Phase III cells.

whether protein misspecification could account for the finite lifetime (Phase III phenomenon) of cultured normal human diploid fibroblasts (WI-38). Quantity of virus produced, pattern of cytopathology, plaque morphology, and analysis of herpesvirus proteins by polyacrylamide gel electrophoresis did not differ in virus progeny produced in young (Phase II) or old (Phase III) WI-38. These results do not support the notion that misspecification of proteins can account for cell aging *in vitro.*

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1. Hayflick, L., and Moorhead, P. S., Exp. Cell Res. **25**, 585 (1961).
2. Hayflick, L., Exp. Cell Res. **37**, 614 (1965).
3. Hayflick, L., Exp. Gerontol. **5**, 291 (1970).
4. Orgel, L. E., Nature (London) **243**, 441 (1973).
5. Martin, G. M., Sprague, C., and Epstein, C. J., Lab. Invest. **23**, 86 (1970).
6. Hayflick, L., Amer. J. Med. Sci. **265**, 433 (1973).
7. Orgel, L. E., Proc. Nat. Acad. Sci. USA **49**, 517 (1963).
8. Orgel, L. E., Proc. Nat. Acad. Sci. USA **67**, 1476 (1970).
9. Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R., J. Biol. Chem. **218**, 607 (1956).
10. Hayflick, L., Tex. Rep. Biol. Med. **23**, 285 (1965).
11. Schneider, E. L., Stanbridge, E. J., and Epstein, C. J., Exp. Cell Res., in press (1974).
12. Reed, L. J., and Muench, H., Amer. J. Hyg. **27**, 493 (1938).
13. Dulbecco, R., and Vogt, M., J. Exp. Med. **99**, 167 (1954).
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).
15. Spear, P., and Roizman, B., J. Virol. **9**, 143 (1972).
16. Holland, J. H., Kohne, D., and Doyle, M. V., Nature (London) **245**, 316 (1973).
17. Baltimore, D., Eggers, H. J., Franklin, R. M., and Tamm, I., Proc. Nat. Acad. Sci. USA **49**, 843 (1963).
18. Holliday, R., and Tarrant, G. M., Nature (London) **238**, 26 (1972).
19. Lewis, C. M., and Tarrant, G. M., Nature (London) **239**, 316 (1972).

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