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EXTENSION OF THE LIFESPAN OF CULTURED NORMAL HUMAN DIPLOID CELLS BY VITAMIN E

Lester Packer and James R. Smith

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July 18, 1974

Classification: Cell Biology

Title: Extension of the Lifespan of Cultured Normal Human Diploid Cells by Vitamin E
(cell aging/vitamin E/WI-38 cells/human diploid fibroblasts/cell pathology)

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Running title: Extension of Human Cell Lifespan by Vitamin E

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ABSTRACT

Inclusion of vitamin E (dl-α-tocopherol) in the culture medium for human diploid cells greatly prolongs their *in vitro* lifespan. The addition of 100 μg of dl-α-tocopherol per ml of medium has allowed us to culture WI-38 cells for more than 100 population doublings to date. (These cells normally have an *in vitro* lifespan of 50 ± 10 population doublings). Cells at the 100th population doubling have a normal diploid karyotype, appear to behave in all other respects like young WI-38 cells and are still actively dividing. We interpret this result as support for the free radical theory of aging.

INTRODUCTION

The finite lifespan of human diploid fibroblasts in culture has attracted attention since the proposal by Hayflick and Moorhead (1,2) that such a system may serve as a model for cellular aging. For example, morphological and biochemical changes have been observed to occur during the lifespan of these cells in culture (3), and electron microscopic examination has revealed an increase in the number of lysosomes, electron dense residual bodies surrounded by membranes, and amorphous insoluble deposits (cf. studies of Cristofalo and Lipetz (4), Robbins et al. (5)). More recently, Deamer (6), using fluorescence microscopy, has demonstrated large increases in diffuse and particulate fluorescent material in cultures near the end of their in vitro lifespan. Evidence invoked to support the hypothesis that cell death in this system is relevant to aging in vivo has come from studies showing an inverse correlation between the age of the donor of skin fibroblasts and the lifespan of these cells in vitro (2,7).

WI-38 cells contain extractable amino iminopropene substances, known to be formed as a consequence of oxidative damage processes that lead to crosslinking reactions (8,9). In this respect, Harman's proposal of several years ago, that free radical-mediated reactions might contribute significantly to the aging process (10), is of interest, particularly in light of recent evidence that antioxidants, known to inhibit free radical-mediated reactions, effectively increase the lifespan of organisms in vivo (11,12). Antioxidants also inhibit the accumulation of lipofuscin (age pigment) in various tissues (13,14,15). Nevertheless, convincing evidence that free radical-mediated reactions are important in aging processes of human cells has not yet been reported. On this basis, we

have chosen to study the effect of vitamin E, a universal, natural antioxidant, on cultured WI-38 embryonic human lung cells. Compared to other systems used to investigate aging processes, the human diploid cell system is relatively simple, and its immediate cellular environment is easily modified. Vitamin E presumably acts to terminate peroxidation reactions in cellular membranes (16), and its basic biological function seems to be to terminate free radical reactions.

MATERIALS AND METHODS

Cell Culture. Starter cultures of WI-38 cells obtained from Dr. Hayflick's laboratory were cultured in Eagle's Basal Medium (Hanks' salt) that contained 10% fetal calf serum (Pacific Biologicals Co., Berkeley, Ca.) and 50 μg aureomycin per ml (Lederle Laboratories). A single lot of fetal calf serum was used throughout these experiments. This lot of serum was screened for viral contamination by Pacific Biologicals Co. and found to be uncontaminated. The culture conditions and subcultivation procedures have been described previously (1,17). 100 mg dl-α-tocopherol, vitamin E, in sealed sterile ampules from Sigma Chemical Co., St. Louis, Mo. (or Eastman Organic Chemical Co.), was added to 100 ml basal medium that contained fetal calf serum and homogenized in a Waring blender for 30 sec. This procedure resulted in a crude suspension of dl-α-tocopherol in the medium which remained turbid for 3 to 4 days. Tocopherol-containing medium was used immediately after preparation. The tocopherol was added directly to the medium in order to avoid possible complications due to the addition of organic solvents to the system. The culture medium was unchanged for one week, after which time the cultures were split 1:4 or 1:2 and were given fresh medium that contained the appropriate concentration of dl-α-tocopherol. At each subcultivation the cells from duplicate flasks

of both control and treated culture were trypsinized and counted in a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.), and the number of cells per cm^2 of growth surface calculated. The population doubling level was estimated by adding 2 population doublings at each 1:4 split or 1 at each 1:2 split. When cultures would no longer grow into a confluent monolayer of cells within three weeks after a 1:2 split when fresh medium was added each week, the cultures were designated as Phase III, i.e., at the end of their lifespan in vitro. Control and experimental cultures were tested periodically for mycoplasma contamination (tests performed by L. Hayflick, Stanford University) and were always found to be negative.

Labeling Index. The percentage of cells synthesizing DNA was determined essentially by the method of Cristofalo and Sharf (13). Cells were planted at 1×10^4 or 2×10^4 cells per cm^2 in Falcon 25 cm^2 flasks, and 0.5 μCi/ml [^{3}H]thymidine (specific activity, 20 Ci/mmole) was added 24 hrs after planting. Thirty hrs after addition of [^{3}H]thymidine, the culture medium was decanted, the cell sheet rinsed twice with Ca^{++}- and Mg^{++}-free phosphate buffered saline, fixed for 5 min with methanol, and washed once with methanol. The cell surface was coated with nuclear track emulsion (type NTB, Eastman Kodak Co.), exposed for four days at 10°C, developed in Kodak D-19 developer, and fixed in Kodak rapid fix. A light stain of hematoxylin was used as a nuclear stain. The percentage of cells that had synthesized DNA was determined by counting at least 200 cells per flask in several fields taken at random over the surface of the flask. If a cell had at least 10 autoradiographic grains over the nucleus, it was scored positive for DNA synthesis (background was 1 to 2 grains per nucleus).

Karyology. Cells were prepared for chromosome analysis according to standard methods (10,18). Karyotypic analysis was performed by Dr. A. Mitchell, Stanford Research Institute, Menlo Park, Ca., and subjected to the test for normal human diploid karyotype set down by the Cell Culture Committee [1969 (19) and 1971 (20) meetings] to determine suitability of human cells for vaccine production.

Thiobarbituric Acid-Reactive Material. The concentration of intracellular lipid peroxidation products was estimated by the reaction of thiobarbituric acid (TBA) with malondialdehyde, according to a modification of the method of Barber (21). Culture medium was removed and the cell sheet was rapidly washed three times with ice cold Ca^{++}- and Mg^{++}-free basal salts. Three ml of 5% trichloroacetic acid were added to the cell sheet. Cells were scraped from the flask and then homogenized in the trichloroacetic acid. To the homogenate, 0.5 ml 0.75% of thiobarbituric was added, the solution was boiled for 15 min., cooled to 4°C and the suspensions centrifuged at 800 × g for 10 min to remove the precipitate. The absorbance of the supernatant at 532 nm was taken as an indication of the presence of thiobarbituric acid reaction material. Total cellular protein was estimated according to the method of Lowry et al. (22).

Detection of Fluorescent Damage Products. Fluorescence microscopy was carried out as described by Deamer (6-8).

Incorporation of dl-α-tocopherol. Dl-α-[^{3}H]tocopherol (Amersham-Searle) was mixed with unlabeled tocopherol (final specific activity, 200 μCi/mg) and added to the culture medium as described above. The [^{3}H]tocopherol-containing medium was added to confluent cultures. After one to two days of treatment, the medium was removed and the cell sheet washed five times with Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution.

The cell sheet was removed by scraping, then sonicated for 1.5 min in 2.5 ml of Ca^{++}- and Mg^{++}-free phosphate buffered saline and an aliquot of the homogenate was centrifuged for 20 min at 800 × g to sediment unbroken cells and large debris. The supernatant was centrifuged 45 min at 100,000 × g. The culture medium, wash solution, cell homogenate, supernatant fractions, and resuspended 100,000 × g pellet were assayed for tritium content in 2 ml of BioSolve (Beckman Instruments, Inc.) to which was added 10 ml scintillation fluid.

Environmental Stress. Visible light treatment - Confluent cultures in 25 cm^2 flasks at the 25th population doubling level were exposed to 1000 foot candles in the visible light range (400-600 nm) at room temperature (23°C) for 20 hrs. During illumination, the growth medium in experimental and control cultures was replaced by Hanks' balanced salt solution with 5% fetal calf serum to eliminate possible photosensitization by the vitamins, amino acids and phenol red dye present in the growth medium. Control cultures were kept at room temperature while the experimental cultures were illuminated. Immediately after illumination, the medium was replaced and the number of cells per flask was determined 24 hrs later.

Growth in the presence of high oxygen concentration - Cultures were prepared as described above except that the caps of the flasks were left loose to permit equilibration of the atmosphere of flasks placed inside desiccators containing higher oxygen concentrations. To prevent medium evaporation, a reservoir of water was used in the desiccators. The appropriate atmosphere was introduced from compressed air tanks (Liquid Air Corp., San Francisco, Ca.) containing either 20, 40 or 60% oxygen, 5% carbon dioxide, and the balance nitrogen.

RESULTS

Cell Proliferation. The effect of dl-α-tocopherol on the long-term proliferation of WI-38 cells in culture is shown in Fig. 1. In parallel subcultivation series (which originated from a single starter culture) in which two different concentrations of tocopherol were employed (100 μg/ml and 10 μg/ml) in the medium, it was observed that the cells grown in the presence of tocopherol from the 45th population doubling level (PDL) consistently had a longer lifespan *in vitro* than the control cells. The control cells reached Phase III at the 65th population doubling while the treated cells were apparently healthy and were still capable of growing in culture despite the fact that they had been subcultivated for more than 100 population doublings (Fig. 1).

After the cultures grown in the presence of 10 and 100 μg tocopherol per ml medium had reached the 73rd population doubling level, tocopherol was removed from the medium and the cells were grown in tocopherol-free medium. These cells have undergone 95 to 100 population doublings and are growing as well as those which have been continuously cultured in tocopherol medium since the 45th population doubling level. Thus, a relatively brief period of growth in tocopherol appears to have a long-term effect on cell proliferation.

From autoradiographic analysis, performed at every second population doubling from population doubling level 85 to 97 on cells continuously exposed to tocopherol, we consistently found that 95 ± 3% of the cells were capable of synthesizing DNA between the 24th and 54th hour after subcultivation. According to Cristofalo and Sharf (23), this result suggests that these cultures have about 70% of their *in vitro* lifespan remaining.

The proportion of cells that attached to the growth surface at subcultivation was determined by trypsinizing and counting the cell suspension with a Coulter counter for control cells and for cells grown in 100 μg tocopherol per ml medium at 8 hrs after planting for four consecutive 1:4 subcultivations. About 40% of the control cells attached, while about 30% of the tocopherol cells attached at each subcultivation, indicating that the increased number of population doublings observed in tocopherol-treated cultures is due to an increased number of cell divisions rather than a decrease in cell loss upon subcultivation.

The growth of cells at the 20th population doubling in various concentrations of tocopherol is shown in Fig. 2. No effect on growth rate or saturation density was observed at the concentrations of tocopherol used in this experiment. However, an increase in the time between subcultivation and the beginning of cell division was observed with 100 and 300 μg tocopherol/ml of medium. Hence at the concentrations of tocopherol used for long term growth experiments little effect on the short term growth of WI-38 cells occurs.

Normalcy of WI-38 Cells with Increased Lifespan. Tocopherol treated cells were subjected to a number of tests after the 85th population doubling level to determine whether they met criteria for normal human cells. Cells which had been continuously treated with 100 μg tocopherol per ml medium exhibited density dependent inhibition of proliferation expected for normal WI-38 cells as shown by the growth curves of tocopherol-treated cells at the 93rd population doubling level and control cells at the 25th population doubling level (Fig. 3).

Tocopherol-treated cells were subjected to karyotypic analysis at the 85th and 100th population doubling level by direct microscopic analysis of

chromosome preparations. In addition, the 100th population doubling level culture was subjected to more stringent analysis, requiring photographic reconstruction of 20% of the chromosome "spreads" examined. Table I summarizes the karyotypic data from these two analyses. Both cultures had a normal human diploid karyotype.

Cells at the 100th population doubling level met all the exacting criteria for normalcy set down by the 1969 meeting of the Cell Culture Committee (19) with modification from the 1971 meeting (20).

Cells from the same subcultivation series were tested for the presence of SV-40 tumor antigen (SV-40 T-antigen) and showed no indication of SV-40 transformation according to this criterion. When tested in parallel with the tocopherol-treated WI-38 cells, cells known to be transformed by SV-40 gave positive T-antigen reactions.

Examination of tocopherol-treated cells by light microscopy after more than 90 population doublings revealed that these cells manifested the same properties as young cells in terms of size, shape and pattern of growth (24) (Fig. 4).

Incorporation of Tocopherol into the Subcellular Fraction. To determine whether tocopherol added to the medium in the manner described above was incorporated into cellular organelles, we followed the fate of tritium-labeled tocopherol mixed with unlabeled tocopherol. About 85% of the tocopherol was lost from the medium because of adsorption to the sides of the flask and formation of a thin film of tocopherol on top of the medium within the first 24 hr. When 100 μg of tocopherol was added per ml of medium, about 0.5% of the tocopherol remaining in the medium was incorporated into cellular organelles (100,000 × g pellet after sonication). This is equivalent to 2-3 μg tocopherol per mg cellular protein.

Protection Against Oxidative Damage by Tocopherol. If the mechanism of dl-α-tocopherol in prolonging cellular lifespan is due to its action as an antioxidant, it should prevent accumulation of oxidative damage to medium constituents or to cellular components. To test this, WI-38 cells were examined for the presence of thiobarbituric acid reactive material, indicative of the formation of malondialdehyde, a hydroperoxide decomposition product of polyunsaturated fatty acids. In a typical experiment control cells showed 0.7 to 1.0 and tocopherol treated cells 0.08 to 0.5 nmoles of thiobarbituric acid reactive material per mg protein. Examination of cells grown in the presence of tocopherol by fluorescence microscopy (Fig. 4) revealed the virtual absence of the fluorescent material shown by Deamer (6) to be associated with non-dividing cells.

Protection by dl-α-Tocopherol Against Environmental Stress. To further evaluate the potential protective action of dl-α-tocopherol on WI-38 cell proliferation, experiments were designed in which the cells were subjected to acute environmental stress by raising the oxygen tension and by illuminating with visible light. It is generally agreed that such environmental stress leads to free radical production and subsequent oxidative damage by peroxidation.

Treatment of WI-38 cells with visible light decreased the number of viable cells in cultures without tocopherol by 90 ± 5% compared to unirradiated cultures. When 1 mg tocopherol per ml medium was present in the balanced salt solution, the decrease in the number of viable cells was only 30 ± 10%. The number of viable cells in control cultures was not decreased by standing at room temperature in Hanks' balanced salt solution + 5% fetal calf serum for 20 hrs.

In other experiments, we observed about 90% inhibition of division rate when cultures were placed in an atmosphere composed of 40% oxygen,

55% nitrogen and 5% carbon dioxide as compared to control cultures grown in 95% air and 5% carbon dioxide. The addition of 1 mg of tocopherol per ml of medium resulted in a partial reversal of this inhibition to about 50% of the value obtained in 20% oxygen without added tocopherol. No cell growth was obtained in an atmosphere of 60% oxygen.

DISCUSSION

Hayflick (1,2) has proposed that normal human fibroblasts in cell culture are capable of a finite number of divisions when cultured in an adequate environment. However, we have been able to grow WI-38 cell cultures to 100 population doublings in the presence of 100 μg of dl-α-tocopherol per ml medium. Throughout the entire course of these experiments WI-38 cells were the only cells cultured in our laboratory, thus eliminating the possibility of contamination by other cell types. At the 100th population doubling level, these cultures still have the normal karyotype and are still growing as rapidly as cultures at the 20th population doubling level. These cultures also exhibit normal density dependent inhibition of growth and morphology. They do not possess the tumor antigen of SV-40, which is the only virus known to permanently transform these cells. The percentage of cells capable of synthesizing DNA is equivalent to cultures with more than 70% of their *in vitro* lifespan remaining (23). According to this criterion these cultures are expected to undergo many more population doublings before reaching Phase III. The experiments reported here suggest that in an appropriate environment, human fibroblasts may be capable of a very large number of divisions, and perhaps an indefinite number.

Previous attempts to extend the lifespan of human fibroblasts *in vitro* utilizing high serum levels in the medium, continuously perfusing the medium

or adding hydrocortisone have had only limited success. In none of these cases does a specific explanation exist for the effect observed (see review by Cristofalo (25)). In one instance, high levels of bovine serum albumin added to the medium increased the lifespan of several strains of fibroblasts, including some of human origin (26); it may be proposed that the bovine serum albumin molecules scavenge free unsaturated fatty acids released from phospholipids. This explanation is consistent with the results reported here and with the direct correlation between dietary vitamin E and unsaturated fatty acid levels (27).

It is generally accepted that tocopherol exerts its protective action by inhibiting oxidative damage. Tocopherol is preferentially partitioned into the hydrophobic areas of membranes where this substance comes in close proximity to the unsaturated fatty chains of lipids to stabilize the membrane (28). The medium in which certain types of human cells are usually cultured may contain inadequate quantities of natural antioxidants, i.e., a vitamin E deficiency may exist. Normal levels of vitamin E in human and bovine plasma are about 15 μg/ml. Our culture medium supplemented with 10% fetal calf serum contains at least 10-fold lower levels of vitamin E. Thus, enrichment of the culture medium with dl-α-tocopherol probably brings the level of this vitamin into the physiological concentration range for plasma. The effectiveness of vitamin E taken up from the medium as an antioxidant in this system is indicated by lower amounts of detectable thiobarbituric acid reactants and protection from the deleterious effects of high oxygen concentration and photosensitized cellular damage.

The action of dl-α-tocopherol in prolonging the lifespan suggests that free radical-mediated peroxidation reactions could damage macromolecules, such as proteins and nucleic acids. We have found evidence that the

oxidative capacity of the mitochondria of WI-38 cells is about twice that needed to maintain cell proliferation (29). In vitamin E deficiency, WI-38 membranes, particularly those of mitochondria, are readily susceptible to peroxidative attack. The spreading of this process in the cytoplasm could interfere with the turnover of cellular materials resulting in an accumulation of indigestible polymers and defective enzymes. Consistent with such views is the diminished accumulation of fluorescent damage products in vitamin E treated cells.

The present investigation suggests that senescence in WI-38 cells is associated with oxidative damage occurring during their proliferation in culture. A balance probably exists between the occurrence of environmentally induced oxidative damage to cells in culture and their ability to neutralize it. It is possible that by decreasing the rate of occurrence of this damage, its gradual accumulation may be prevented, thus allowing prolonged cellular proliferation.

Since tocopherol confers upon WI-38 cells a long-term ability to proliferate in culture, these WI-38 cells provide a unique model system for evaluating environmental effects (30,31,32) on the growth potential of human cells. In vivo accumulated damage to cells may account for the inverse relationship between the age of the human donor and the lifespan of skin fibroblasts (7). In addition, the increased in vitro lifespan should increase the probability of obtaining stable mutants and chemical transformation in these cells.

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TABLE 1. Karyotypic analysis of WI-38 cells

Cultures	Frequency (cells per 100)						
	Hyper-diploid	Hypo-diploid	Polyploidy	Chromosome breaks	Chromatid breaks	Structural abnormalities	Other abnormalities
PDL 85	0	6	0	0	5	2	0
PDL 100	1	16	0	1	1	1	0
Maximum acceptable number*	2	18	4	8		2	At discretion of control authority

*References 19 and 20.

PDL = population doubling level.

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Figure Legends

Fig. 1. Effect of dl-α-tocopherol on *in vitro* proliferation of WI-38 human diploid lung fibroblasts. A. Cumulative population doublings in the continuous presence of vitamin E. Control cultures, ●; cultures treated continuously from the 45th population doubling level with 10, ▲; or 100 μg tocopherol/ml medium, o.

Fig. 2. Growth of cells at the 20th population doubling level in various concentrations of dl-α-tocopherol. 2.5×10^5 cells/25 cm^2 flask were planted on day zero. Duplicate flasks at each concentration were counted each day. The medium was not changed during this experiment. —o—, control; —●—, 10 μg/ml; —Δ—, 100 μg/ml; —▲—, 300 μg/ml.

Fig. 3. Comparison of growth rate and saturation density of control and vitamin E (100 μg/ml) treated cells. 2.5×10^5 cells were planted on day zero. The medium was replaced with fresh medium every three days. Each point represents the average of two 25 cm^2 flasks.

Fig. 4. Morphology and fluorescence of control and vitamin E treated cells. Magnification 250×. A. Control cells at the 20th population doubling level. B. Control cells at the 53rd population doubling level. C. Cells at the 90th population doubling level continuously treated with 100 g tocopherol per ml medium. D. Fluorescence in control cells at 25th population doubling level. E. Fluorescence in control cells at the 50th population doubling level. F. Fluorescence in tocopherol treated (100 μg/ml) cells at the 97th population doubling level. Cells were grown for two weeks without aureomycin. Photomicrographs were taken 8 days after a 1:4 split. A, B, and C cells were fixed in methanol and stained with Giemsa stain.

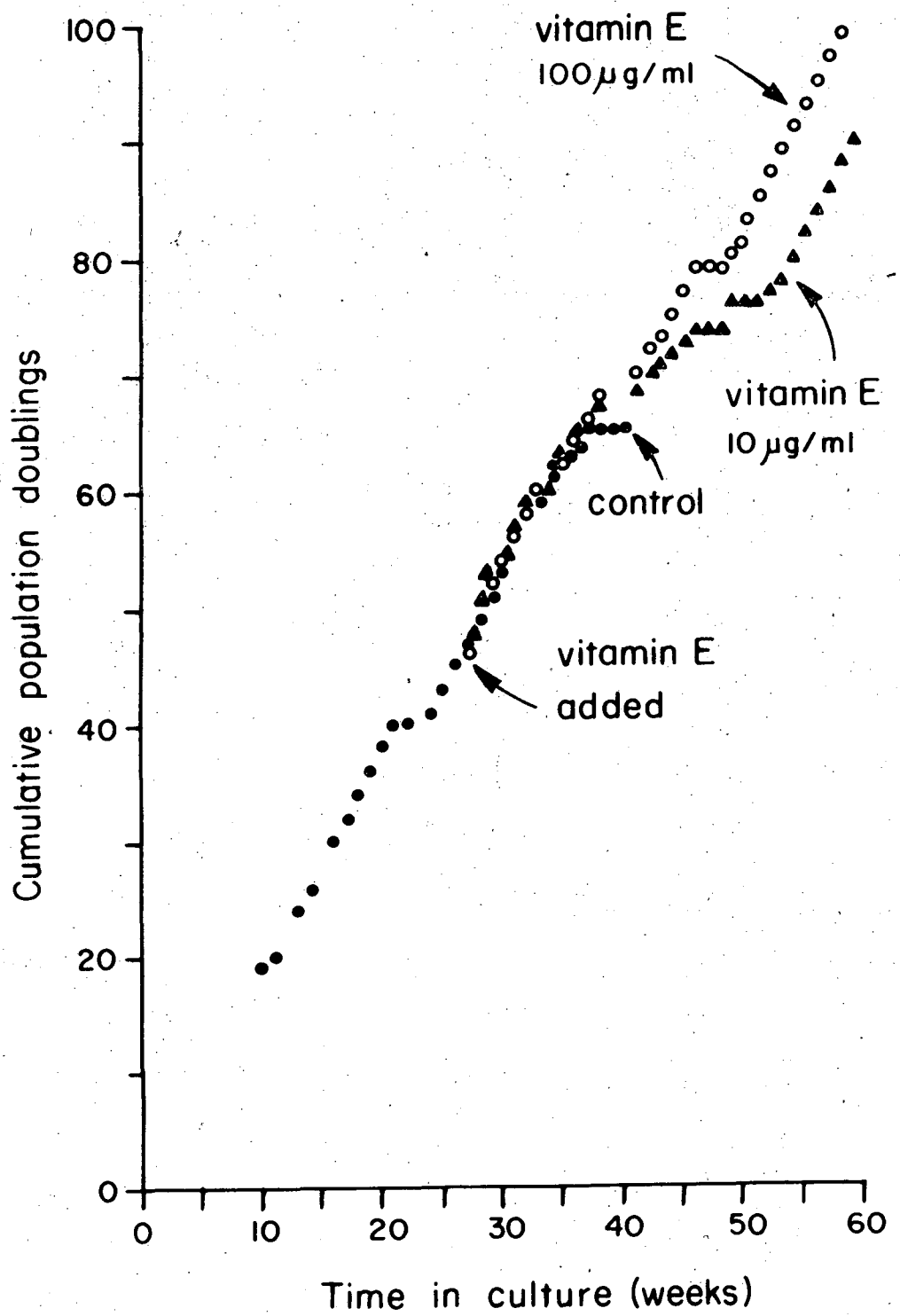

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Fig. 1.

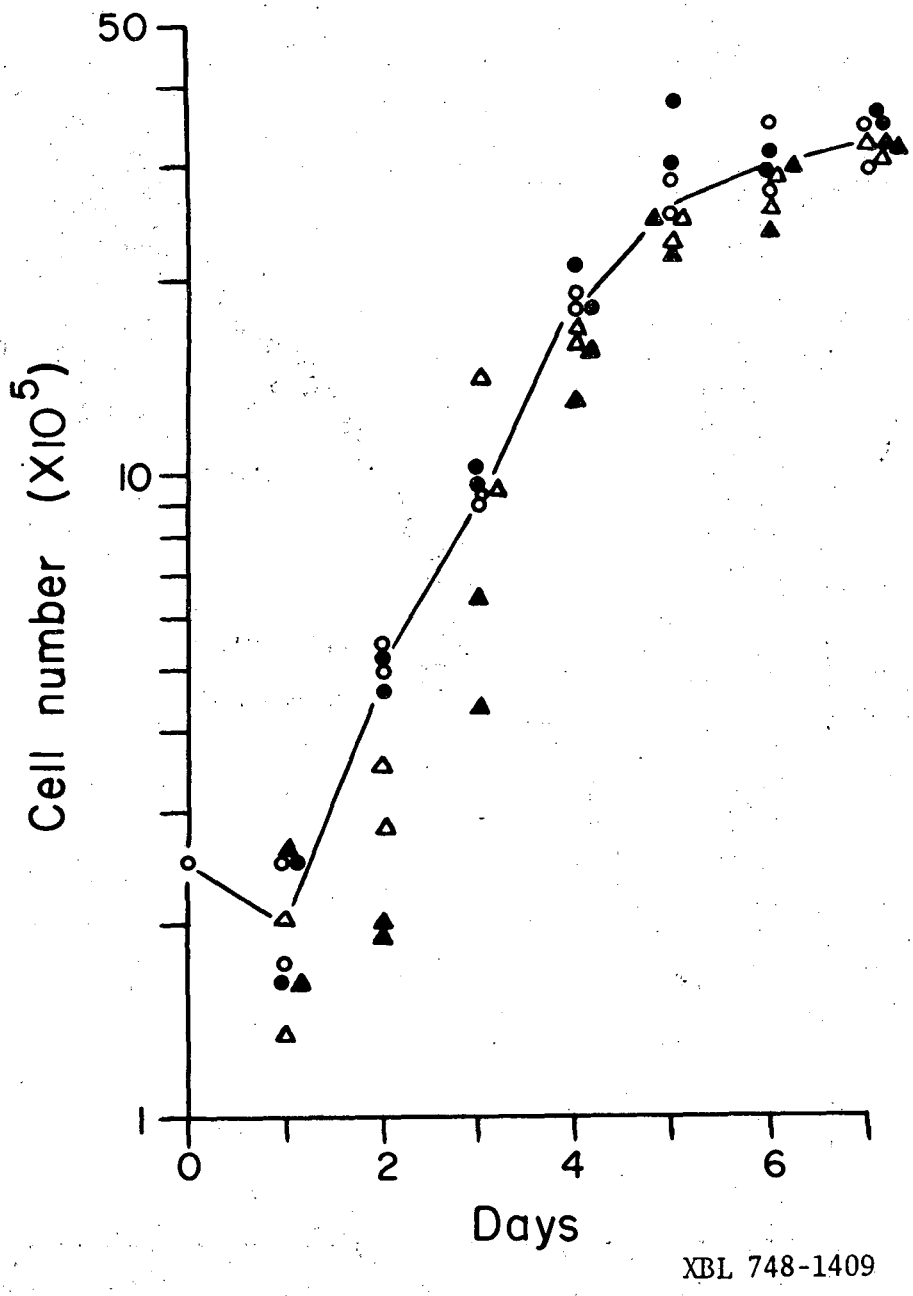

Fig. 2.

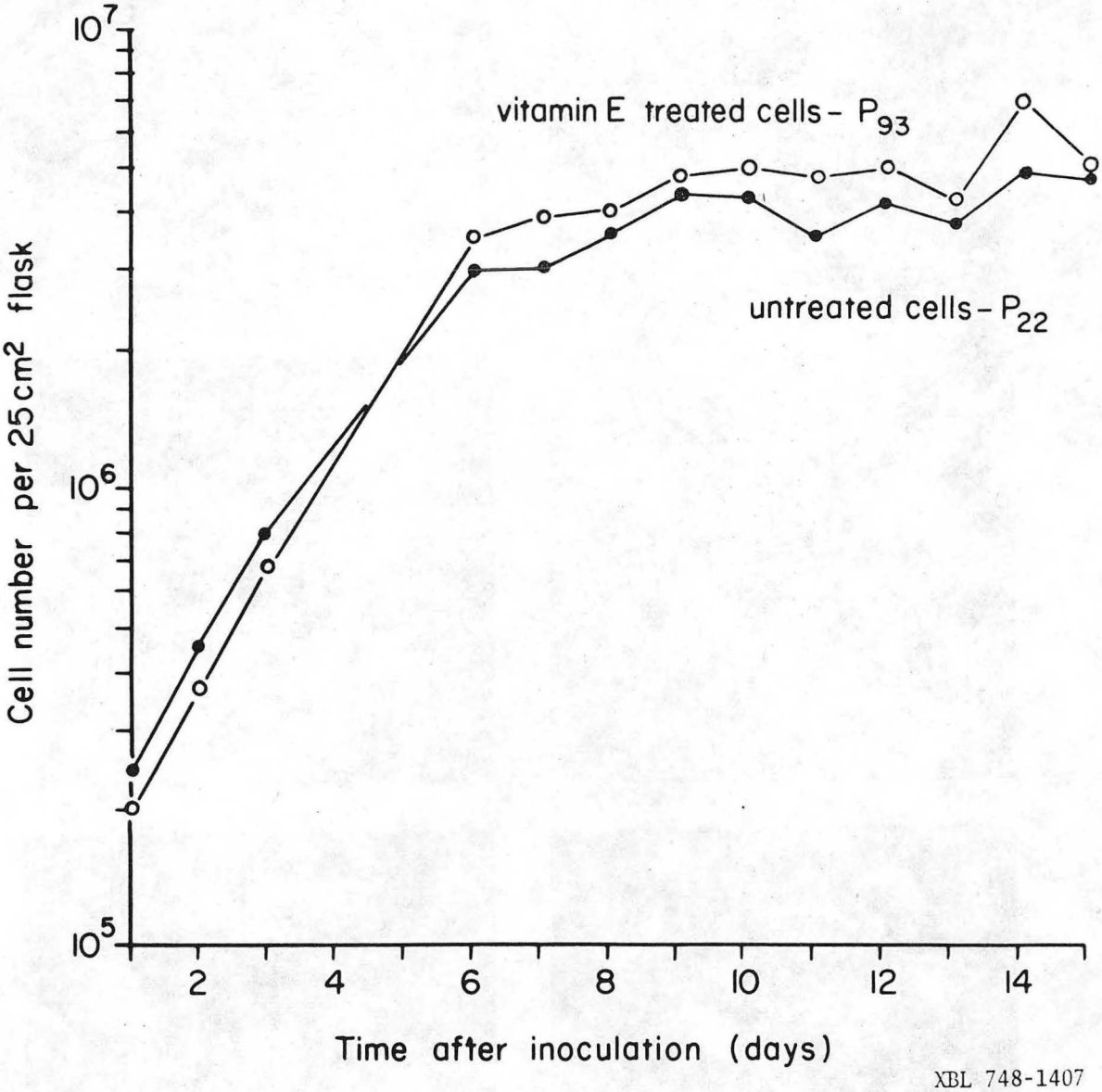

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Fig. 3.

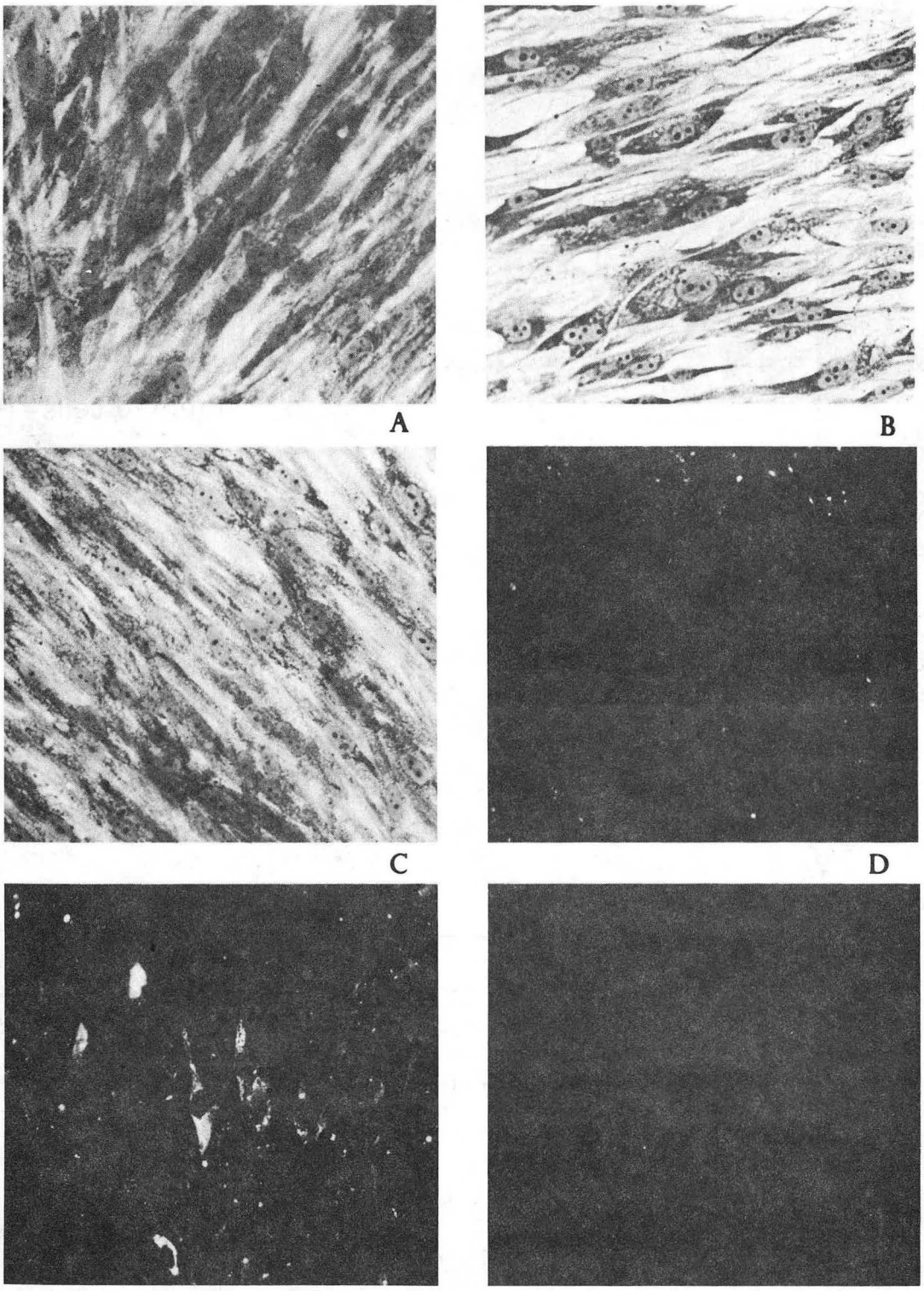

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Fig. 4.

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