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Retardation of the Senescence of Cultured Human Diploid Fibroblasts by Carnosine

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We have examined the effects of the naturally occurring dipeptide carnosine (β-alanyl-L-histidine) on the growth, morphology, and lifespan of cultured human diploid fibroblasts. With human foreskin cells, HFF-1, and fetal lung cells, MRC-5, we have shown that carnosine at high concentrations (20–50 m*M*) in standard medium retards senescence and rejuvenates senescent cultures. These late-passage cultures preserve a nonsenescent morphology in the presence of carnosine, in comparison to the senescent morphology first described by Hayflick and Moorhead. Transfer of these late-passage cells in medium containing carnosine to unsupplemented normal medium results in the appearance of the senescent phenotype. The serial subculture of cells in the presence of carnosine does not prevent the Hayflick limit to growth, although the lifespan in population doublings as well as chronological age is often increased. This effect is obscured by the normal variability of human fibroblast lifespans, which we have confirmed. Transfer of cells approaching senescence in normal medium to medium supplemented with carnosine rejuvenates the cells but the extension in lifespan is variable. Neither *D*-carnosine, (β-alanyl-D-histidine), homocarnosine, anserine, nor β-alanine had the same effects as carnosine on human fibroblasts. Carnosine is an antioxidant, but it is more likely that it preserves cellular integrity by its effects on protein metabolism.
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INTRODUCTION

The dipeptide carnosine (β-alanyl-L-histidine) is widely distributed in tissues and is present in muscle at a particularly high concentration (~20 m*M*). Its function is not known, but claims have been made that it is a neurotransmitter [1], that it activates ATPases [2], and that it has antioxidant activity [3, 4, 5]. It has been shown to protect bacteriophages from the lethal effects of ionizing irradiation [6]. More recently, it has been proposed that it may be active in preventing the nonenzymatic glycosylation of proteins [7, 8].

We have used human diploid fibroblasts to investigate the effects of carnosine on their long-term growth. It is well established that these cells have finite proliferative potential [9, 10]. After a long period of active cell division *in vitro*, the growth rate slows down, the cells fail to line up in parallel arrays, and eventually do not produce a confluent monolayer. Hayflick [10] proposed that human fibroblasts provide a convenient experimental system for studying aging at the cellular level. He defined the final stages of growth as Phase III, when the cells have a characteristic senescent phenotype. Phase I is the establishment of the primary culture, and Phase II is the long period of proliferation when the cells have a normal or nonsenescent phenotype.

We have examined the effect of carnosine on the morphology, growth, and longevity of human diploid fibroblasts, strains MRC-5 and HFF-1, in experiments spanning nearly 3 years. In one experiment cells were grown continuously for more than 2 years. We show that carnosine retards the senescence of late-passage fibroblast cultures and can rejuvenate senescent cells. It also extends chronological lifespan. There is often, but not invariably, an increase in population doublings (PDs) when fibroblasts are grown continuously in medium containing carnosine. It is well known that the lifespans of clones and populations of human diploid fibroblasts are very variable [11, 12]. Therefore, to demonstrate environmental effects on longevity in PDs usually requires the study of many parallel populations, which we have not so far undertaken. Our results confirm, in fact, the stochastic variation in PDs in both MRC-5 and HFF-1. We have also shown that D-carnosine does not have the same effects as the natural dipeptide. In the description of our results, we follow Hayflick's definitions and refer to cells being either senescent or nonsenescent.

MATERIALS AND METHODS

Cells. Human fetal lung strain MRC-5 was obtained at passage 12 from the National Institute of Biological Standards of Control, South

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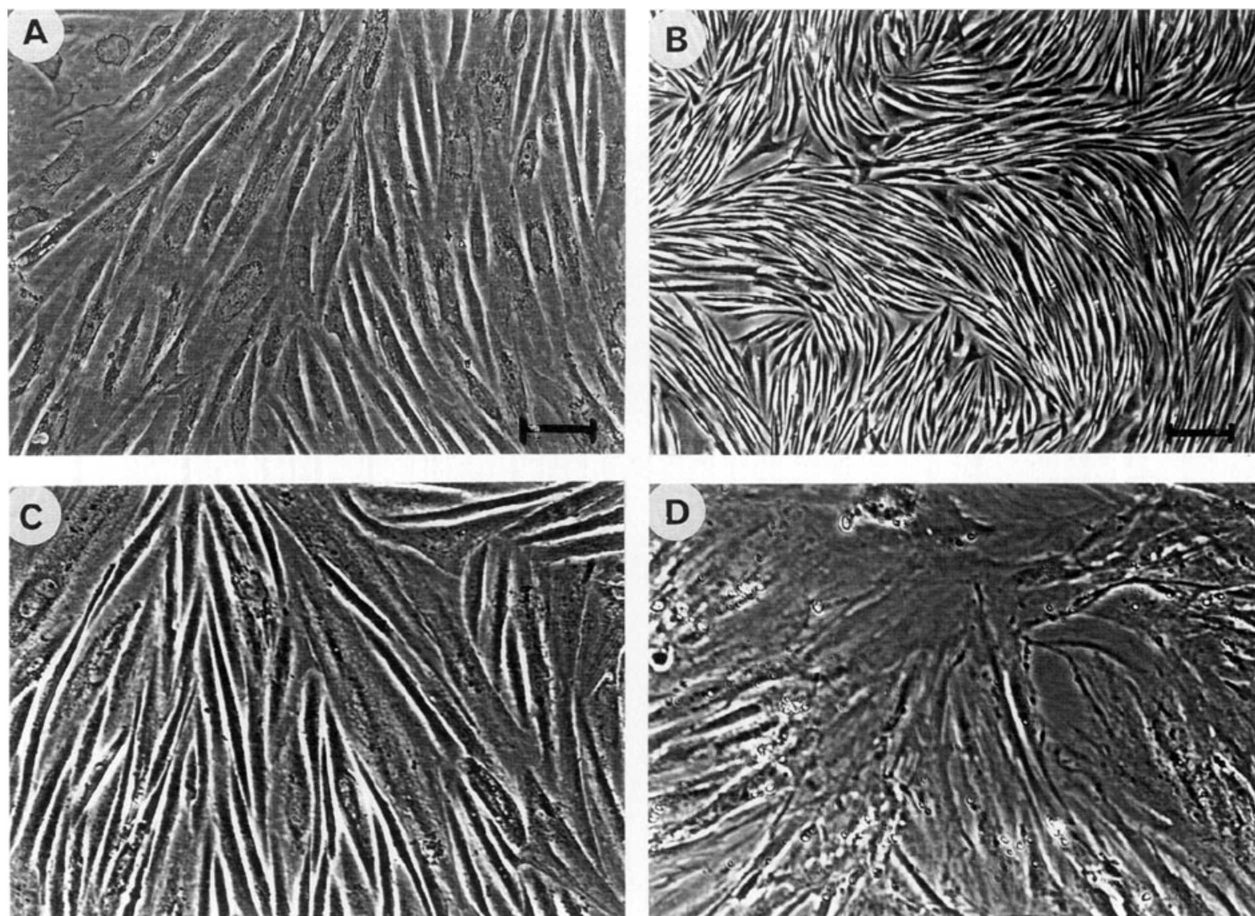

FIG. 1. HFF-1 cells grown in DMEM containing 50 m*M* carnosine. (A) Cells at PD 36.2. (B) Cells at PD 29.1 (low magnification). (C) Cells at PD 47.1. (D) Cells at PD 47.1 transferred to unsupplemented DMEM, 8 days later. Bars 100 μm (A, C, D) and 200 μm (B).

Mimms (Herts., UK). Human foreskin cells, HFF-1, were obtained at passage level 5 from Dr. Roger Reddel, Childrens Medical Research Foundation (CMRF), Camperdown, NSW 2006 (present address: CMRF, Westmead Hospital, NSW 2145).

Cell culture. Cells were grown either in Dulbecco's modification of Eagle's minimum essential medium (DMEM, supplied by Flow or GIBCO), containing 0.45% glucose, supplemented with 10% fetal calf serum and with 60 μg/ml penicillin and 100 μg/ml streptomycin, or Eagle's minimal essential medium (MEM, supplied by GIBCO), containing 0.1% glucose, with the supplement plus nonessential amino acids (Cytosystems Pty., Ltd.). Cells were grown in 25-cm^2 flasks and incubated at 37°C with 5% CO_2. They were harvested with trypsin/versene, and single cell suspensions were counted with a model ZF6 Coulter Counter. HFF-1 cultures were split in ratios of 1:2 and 1:4 and MRC-5 cultures in ratios of 1:2, 1:4, or 1:8. (To equate PDs with passage level, these ratios represent 1, 2, and 3 passages, respectively. Note that young cultures obtained from frozen ampoules are usually labeled with their passage level.) The medium was changed weekly when cultures were not confluent, unless otherwise stated. The cumulative PDs were calculated from the increase in cell number at each subculture. Cultures were discarded when they failed to reach confluence after at least 3 weekly changes of medium. Under Results, chronological age is to the date of the last split. L-Carnosine was obtained from the Sigma Chemical Company. D-Carnosine (β-alanyl-D-histidine) was kindly provided by Peptide Technology Pty., Ltd. (Dee Why, NSW 2099). (Note that in the text L-carnosine is referred to as carnosine.) L-Homocarnosine, L-anserine and β-alanine were obtained from the Sigma Chemical Company. Stock solutions of 100 m*M* were made up in complete growth medium, filter sterilized, and diluted as required. In one early experiment mycoplasma contamination was detected, although this had no discernible effect on growth rate or the cell yield in confluent flasks. These cells were treated with Mycoplasma Removal Agent (MRA) obtained from ICN or as a gift from Dr Toshiharu Matsumura, Meiji Cell Technology Center, Odawara, Japan. The absence of mycoplasma was subsequently confirmed at intervals using the method of Chen [133].

Photomicroscopy. A Nikon Diaphot inverted phase-contrast microscope was used with a Nikon HFX-11 camera attachment. Photographs were taken using Kodak technical pan film.

RESULTS

Initial experiments were carried out with HFF-1, which grows more slowly than MRC-5, and all cultures

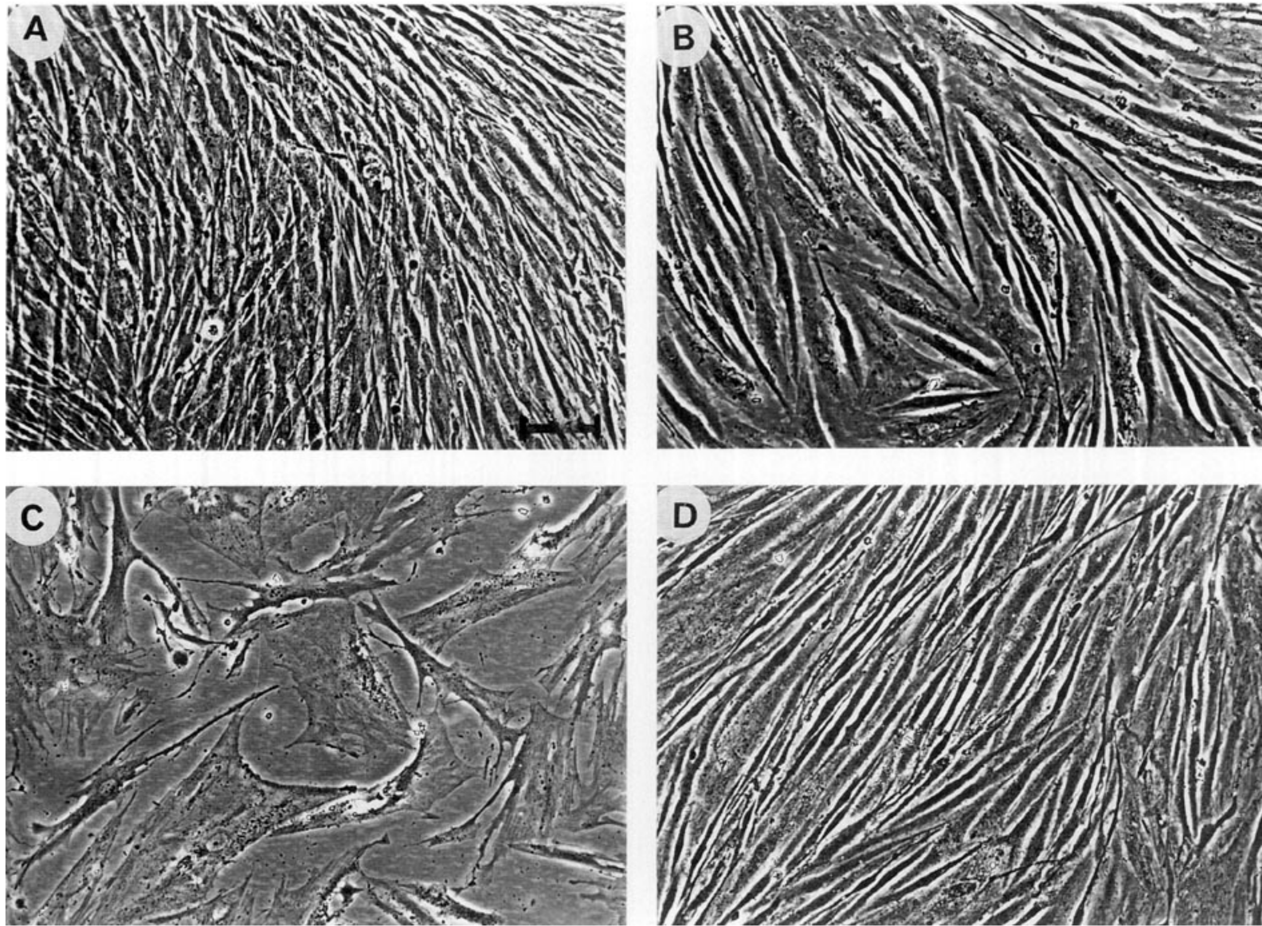

FIG. 2. HFF-1 cells. (A) Control cells grown in DMEM at PD 37. (B) Cells grown in DMEM containing 30 m*M* carnosine at PD 35.4. (C) Senescent control cells grown in MEM at PD 47.2. (D) Cells grown in MEM containing 20 m*M* carnosine at PD 48.5. Bar, 100 μm.

were split with 1:2 and 1:4 ratios. In DMEM supplemented with carnosine, it was found that 10 m*M*, 20 m*M*, and 30 m*M* had little effect on the yield of confluent cells, although the time to reach confluence was significantly reduced at 30 m*M*. Cultures were usually split every 10–12 days with an intervening change of medium. A much greater reduction in growth rate was seen with 50 m*M* carnosine. These cells were split every 2–3 weeks, with a weekly change of medium. The control, 30 m*M*, and 50 m*M* cultures were grown to the end of their lifespan.

The cells grown in DMEM with 50 m*M* carnosine had a very distinctive morphology, as shown in Figs. 1A, 1B, and 1C. In comparison to control cells, those grown in carnosine have a flat, spread-out appearance, with very uniform spacing of cells. These appear to be large, but this was not confirmed after trypsinization (volume estimated in the Coulter Counter). These cells continued to grow with unaltered morphology for most of their lifespan, which lasted 716 days from the start of treatment to the last split. Cells at PD levels 36.2 and 29.1 (high and low magnification) are shown in Figs. 1A and 1B, and growth ceased at PD level 48.7 (Table 1). The strong effect of carnosine in maintaining a nonsenescent morphology at late PD level is shown in Fig. 1C when late-passage cells were transferred to normal DMEM. These cells showed a senescent phenotype after 6–8 days incubation (Fig. 1D). Cultures grown in 30 m*M* carnosine grew to a higher PD level than the controls, and their chronological lifespan was extended (Table 1). These cells had a morphology intermediate between control cells and those grown in 50 m*M* carnosine (Figs. 1A, 2A, and 2B).

HFF-1 cells were also grown in MEM containing 0.1% glucose, and it was observed that carnosine had a significantly greater inhibitory effect than in DMEM (0.45% glucose). In 20 m*M* carnosine, the cells divided more slowly than controls after subculture, but continued to

TABLE 1

The Longevities of Populations of HFF-1 Grown in the Presence of 20, 30 or 50 mM Carnosine, or D-Carnosine

Medium	Treatment	Time of transfer to carnosine	Lifespan	
			PDs	Days
DMEM	Control	—	57.2	432
DMEM[a]	Control	—	52.0	391
DMEM	30 mM carnosine	passage 13	60.2	522
			63.5	564
			61.5	649
DMEM	50 mM carnosine	passage 10	48.7	716
MEM	Controls	—	49.4	310
			47.4	310
MEM	20 mM carnosine	passage 9	56.8	400
	20 mM D-carnosine	passage 9	50.8	289

[a] These cells were initially grown in MEM and then transferred to DMEM at passage 29.

divide for a longer period of time than the control, and the final yield of cells after 10–12 days was the same (results not shown). Cells were grown in MEM, MEM with 20 mM carnosine, and also MEM with 20 mM D-carnosine to the end of their lifespan. The morphology of the cells grown in carnosine was distinct from the control, as the cells approached the end of their lifespan (Figs. 2C and 2D). In this experiment when the control cells became senescent and ceased growth, the cultures in 20 mM carnosine continued to produce a high yield of cells per flask and finally achieved a lifespan 7.4 and 9.4 PDs greater than the controls (Fig. 3 and Table 1). The culture supplemented with 20 mM D-carnosine did not have altered morphology and had almost the same lifespan as the longer-lived control (Table 1). Experiments were also done with HFF-1 cells grown in DMEM containing 30 and 50 mM D-carnosine. These cells did not have the characteristic morphological changes which were seen in medium containing carnosine.

MRC-5 is a faster growing and better characterized strain than HFF-1. Striking effects on the cell morphology of MRC-5 were again induced by carnosine, which were very similar to those seen in HFF-1 cultures (Fig. 4). The first experiment demonstrated the same doubling rate of control cultures and those growing in 20 and 30 mM carnosine (Fig. 5). The cells grown continuously in 20 mM carnosine achieved a lifespan of 70.7 PDs, which was 10 and 14 PDs greater than two control cultures (Table 2). Cells grown continuously in 30 mM carnosine had a lifespan 4 and 8 PDs greater than the controls (Table 2).

In this experiment control MRC-5 cells at PD levels 55.3 and 55.1, which were showing characteristic signs of senescence, were transferred to medium containing 20 or 30 mM carnosine, respectively. These cultures showed a remarkable rejuvenation, with regard to their morphology, as shown in Fig. 6. They continued to grow slowly for an additional 274 days and finally reached PD levels significantly greater than the control culture from which they were derived (Table 2). The cells transferred to 30 mM carnosine had the morphology shown in Figs. 6C and 6D, but the cell yield gradually declined over a 220-day period. However, at this time the cells began to grow faster and the cell yield increased (Fig. 7). This result suggests that a faster growing clone was selected and took over the culture until cell numbers again declined and growth ceased.

A number of further experiments have been carried out with MRC-5. In one series, cells growing in DMEM at passage 51 were transferred to carnosine, L-homocarnosine, L-anserine, and β-alanine (all at 20 mM). (Histidine is very toxic even at 10 mM.) Only carnosine had strong effects on cell morphology. All these cultures were grown to senescence and no striking effects on longevity were seen.

In other experiments, the longevities of MRC-5 cells grown in DMEM with 20 or 30 mM carnosine were variable, but it should be noted that two of these controls reached 79.1 and 76.4 PDs, which are much higher than the usual range of MRC-5 (Table 2). Such long-lived cultures can obviously obscure any effects of carnosine in increasing lifespan. All the populations of MRC-5 grown in carnosine preserved a nonsenescent morphol-

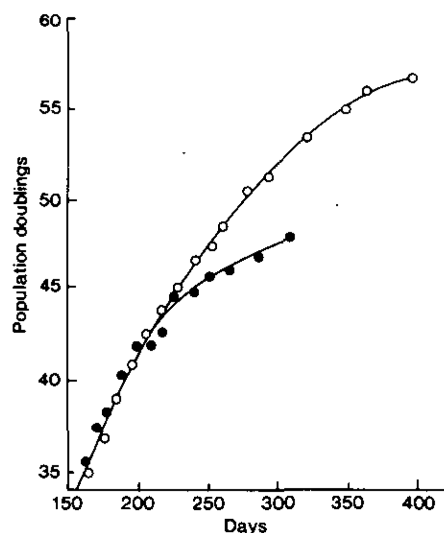

FIG. 3. HFF-1 cells grown in MEM (●) and MEM containing 20 mM carnosine (○). Only the later part of the cumulative growth curve is shown and only one control. The data are from the last experiment in Table 1.

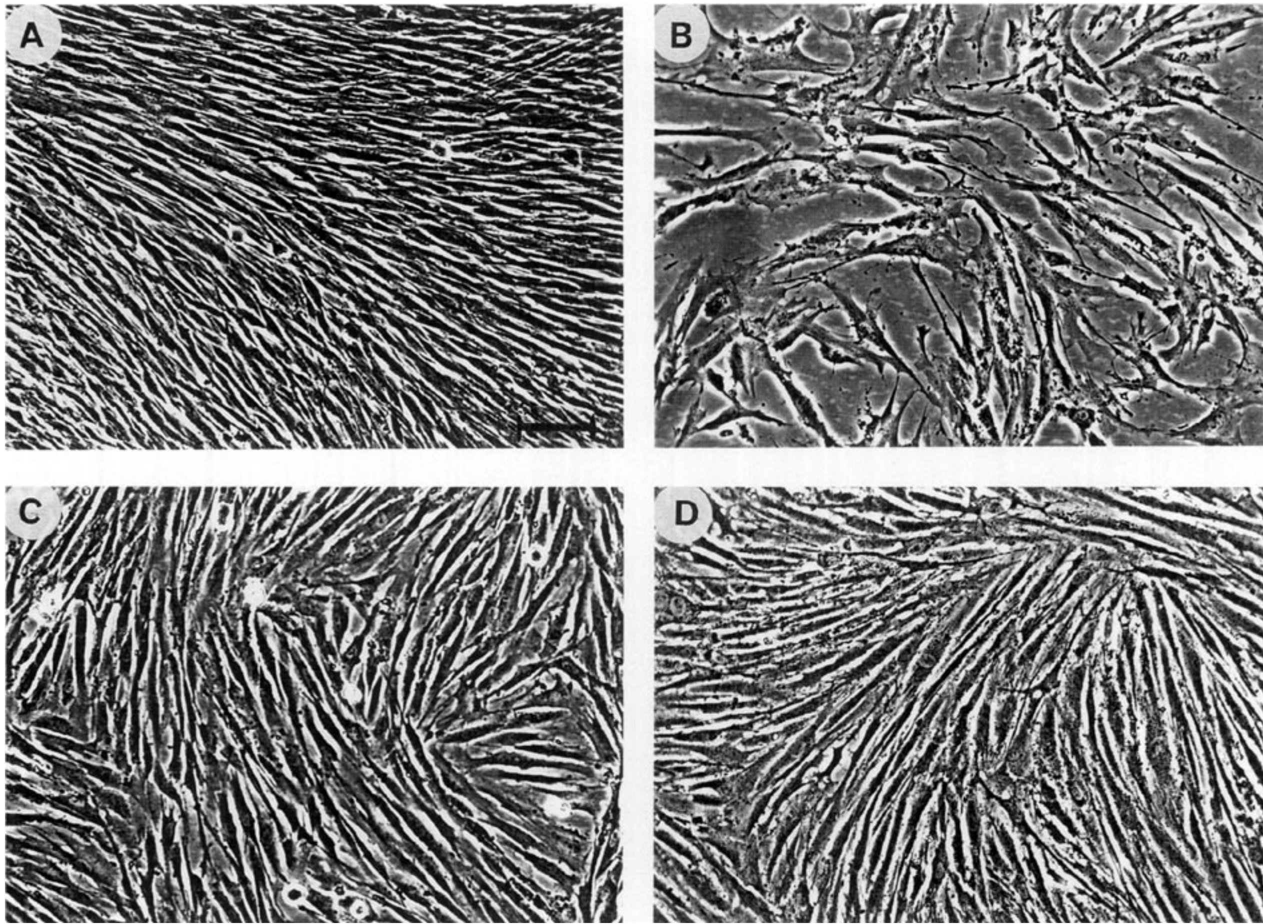

FIG. 4. MRC-5 cells grown in DMEM unsupplemented or with 20 or 30 m*M* carnosine. (A) Control cells at passage 14. (B) Control cells which are senescent at PD 55.1. (C) Cells grown in 20 m*M* carnosine at PD 60.6. (D) Cells grown in 30 m*M* carnosine at PD 60.9. Bar, 100 μm.

ogy until the end of their lifespan, when control cells were showing marked signs of senescence. Also, most of the carnosine-treated cultures grew for a longer period of time than the controls. Late-passage control cells which were becoming senescent when transferred to DMEM containing 20 or 30 m*M* carnosine were rejuvenated, but the extension of growth was also variable. It is possible that carnosine has a strong rejuvenating effect if added before the population reaches a given level of senescence, but not after this. It is hard to compare different experiments when the longevities of cultures are variable.

Experiments were also carried out using MEM medium with and without carnosine. The same morphological changes were induced by carnosine, as in HFF-1 cells, and the appearance of senescent cells was delayed. As in the case of the HFF-1 cells, the cell yield in late-passage flasks was maintained in the populations grown in carnosine, at a time when the number of cells in control flasks was declining. For the last 10 splits the average number of cells was increased by 44 and 51% over the controls in the two experiments. (A similar effect was not seen in the experiments using DMEM, in which the high glucose level generally increased cell yield throughout the whole lifespan.) Also, transfer of control cells at late passage to medium containing 20 m*M* carnosine, rejuvenated the cells with regard to morphology, cell yield, and lifespan in PDs (Table 2).

In all the experiments with carnosine, the cells which had ceased growth had a nonsenescent or less senescent morphology than late-passage cells in unsupplemented medium. In our experience control senescent cells become very granular and also vary in size and morphology (Figs. 2C and 4B). Debris accumulate in the medium as cells detach from their substrate. When late-passage cells in medium containing carnosine fail to become confluent, they nevertheless retain nonsenescent morphology, especially in 50 m*M* carnosine (Fig. 8), al-

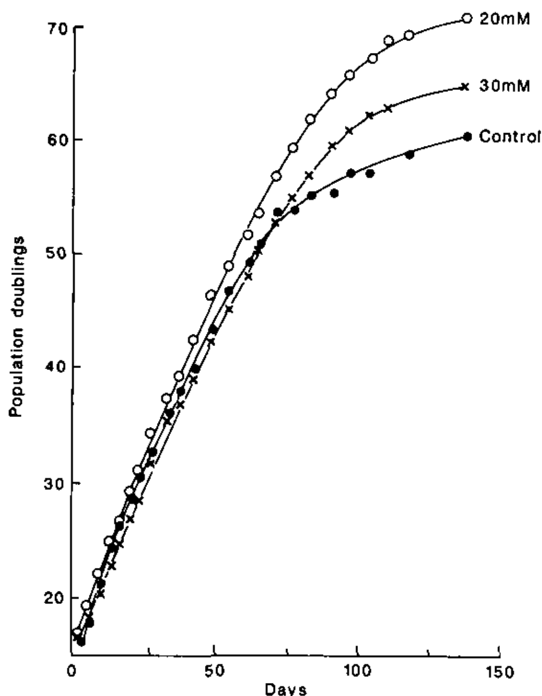

FIG. 5. The cumulative growth of MRC-5 cells in DMEM unsupplemented or with 20 and 30 m*M* carnosine. Cultures were split weekly. Control cells were transferred to medium containing 20 and 30 m*M* carnosine at PD 55.3 and PD 55.1, respectively (see Table 2, Figs. 4 and 6).

though there is some variation in size. These cells do not detach and after many changes of medium a confluent culture may result. This is in contrast to untreated cells which are senescent and subconfluent. In this case, the cells either slowly become confluent after 2–3 weekly changes of medium or they will never do so.

The results in this study confirm the variability of lifespans of populations of MRC-5. In all control experiments ($n = 16$), the range was 56.7–79.2 PDs. With the addition of 20 m*M* carnosine ($n = 12$), the range was 61.3–75.8 PDs, with an average increase of 3.6 PDs, which represents a 12-fold increase in cell mass. Carnosine also extends the chronological lifespan of human fibroblasts, particularly at high concentrations.

DISCUSSION

It was established by Hayflick and Moorhead [9, 10] that human diploid fibroblasts grown *in vitro* eventually reach a senescent condition (designated Phase III) after a long period of normal growth (Phase II) from the establishment of the primary culture (Phase I). Phase III cells have characteristic morphological changes, including a failure to line up in parallel arrays and form normal whorls of growth, irregularities in the size and shape of cells, accumulation of granular material in the cytoplasm, and cell debris in the medium. The major effect of carnosine is to alter these signs of senescence. MRC-5 and HFF-I cells grown in high concentrations of carnosine (20–50 m*M*) preserve a much more normal morphology as they reach the end of their lifespan. Although the cells eventually fail to reach confluence, they do not show the characteristic features of control senescent cells (see especially Figs. 8B, 2C, and 4B). They retain this rejuvenated appearance for a long period, provided the medium containing carnosine is changed weekly. When untreated cells enter Phase III the yield of cells per flask continually declines [10], but we have found that the decline is delayed when medium containing a low level of glucose (MEM, 0.1% glucose) is supplemented with 20 m*M* carnosine. We also find that the cells are more resistant to high levels of carnosine in DMEM (4.5% glucose) than in MEM. We have not seen the same effects of carnosine on morphology when cells were grown at the same m*M* concentrations of D-carnosine, L-homocarnosine, L-anserine, and β-alanine. The other rejuvenating effects of carnosine we have seen are more variable. In some experiments the lifespan in PDs is very significantly increased, but this is not a consistent result. The transfer of senescent cells in normal medium to medium containing carnosine rejuvenates their morphology, but the extension of lifespan in PDs is not predictable.

TABLE 2

The Longevities of Populations of MRC-5 Grown in DMEM or MEM with the Addition of 20 or 30 m*M* Carnosine

Medium	Treatment	Transfer to carnosine (PD or passage level)	Lifespan	
			PDs	Days
DMEM	Controls	—	56.7	126
			60.6	139
DMEM	20 m*M* carnosine	Passage 14	70.7	139
			69.7[a]	139
DMEM	30 m*M* carnosine	Passage 14	64.7	139
			64.3[a]	139
DMEM	20 m*M* carnosine	55.3 PDs	69.7	413
DMEM	30 m*M* carnosine	55.1 PDs	68.8	413
DMEM	Controls	—	79.2	181
			76.4	152
DMEM	20 m*M* carnosine	Passage 16	75.8	203
			75.8	203
MEM	Control	—	61.8	162
MEM	20 m*M* carnosine	50.2 PDs	65.3	175

[a] Duplicate 20 m*M* carnosine cultures were set up at 59.3 PDs and duplicate 30 m*M* cultures at 54.8 PDs. The other duplicated cultures were set up at the time of transfer to carnosine.

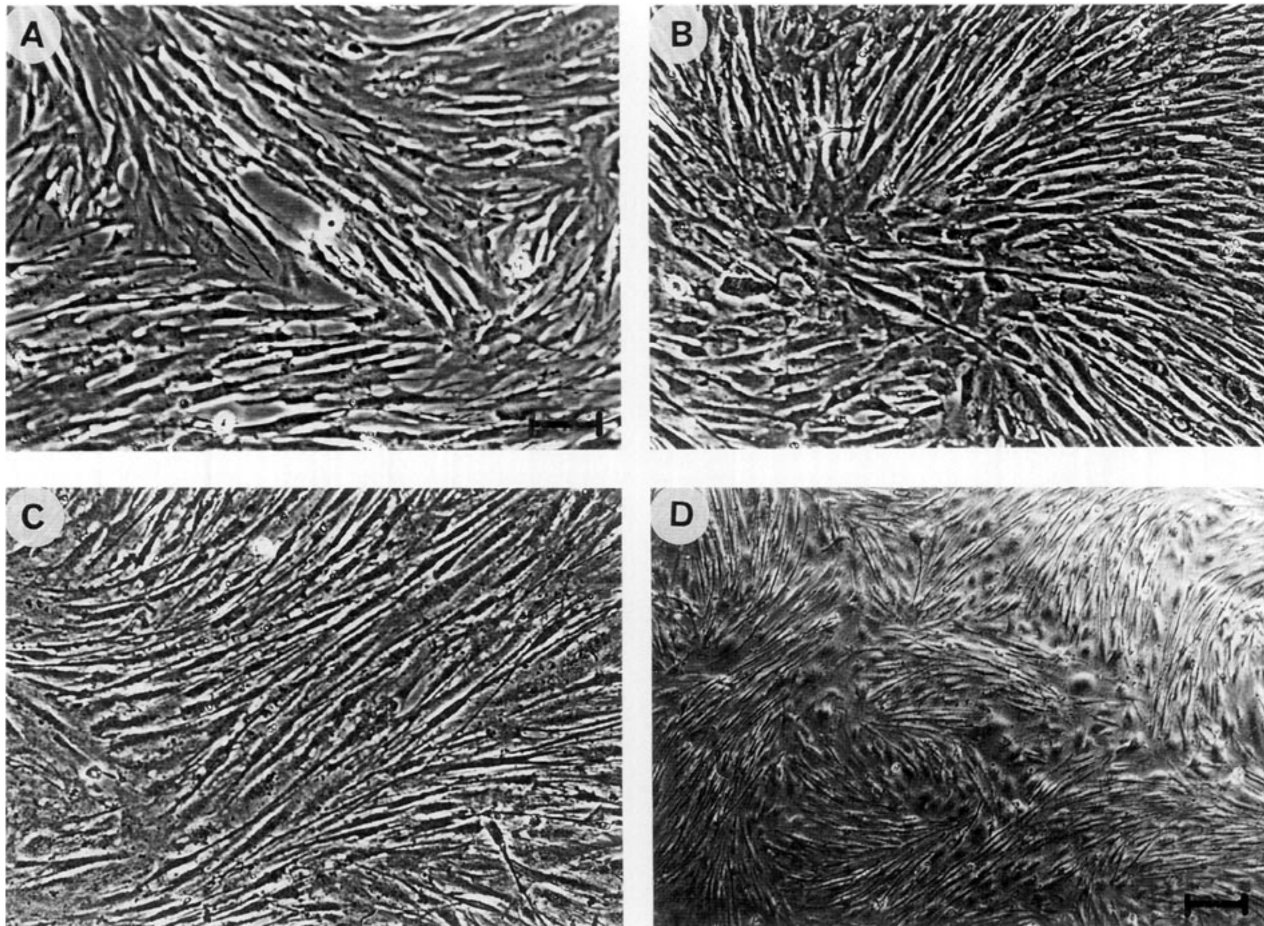

FIG. 6. MRC-5 cells grown in DMEM and transferred at late passage to medium containing carnosine. (A) Control at PD 55.3; signs of senescence are visible. (B) Cells transferred at PD 55.3 to medium containing 20 m*M* carnosine at PD 65.3, 159 days later. (C) and (D) Cells transferred at PD 55.1 to medium containing 30 m*M* at PD 66.6, 230 days later, normal magnification (C) and low magnification (D). (See Table 1, Figs. 5 and 7.) Bar, 100 μm (A–C) and 200 μm in D.

It is possible that the longevity results can be explained on the basis of the commitment theory of cellular aging [12, 24, 15]. This proposes that cells which are not committed to future senescence are present in early-passage populations, but these cells continually give rise to committed cells which have a finite lifespan. During routine subculture the uncommitted cells soon become diluted out, but the fate of the culture in terms of its final lifespan, depends on the stochastic loss of these few remaining uncommitted cells. The theory is able to account for the statistical variation in lifespans of both W1-38 and MRC-5 cells [12]. It also predicts that in late-passage cells there can be clonal succession of specific populations, and the final population may be a single clone. Experiments with mixed populations of human fibroblasts [16] or human T lymphocytes [17] support this prediction. Our experiments with carnosine provide indirect support for the commitment theory. They suggest that carnosine may influence early events in the growth of the culture, for example, the likelihood of loss of uncommitted cells, and this can result in an extension of lifespan in PDs. In this connection it may be significant that the strongest effect of carnosine was seen in the experiment which started with the earliest-passage MRC-5 cells.

The commitment theory does not address the problem of the actual mechanism of senescence, and one interesting possibility is that commitment is the loss of DNA telomerase. The cells would ultimately die when they lose sufficient telomeric DNA [18, 19]. Treatments which increase lifespan may require some modification of the loss-of-telomeres theory of cellular aging, at least in its simplest form. Apart from carnosine, other treatments which can increase fibroblast lifespan are hydro-

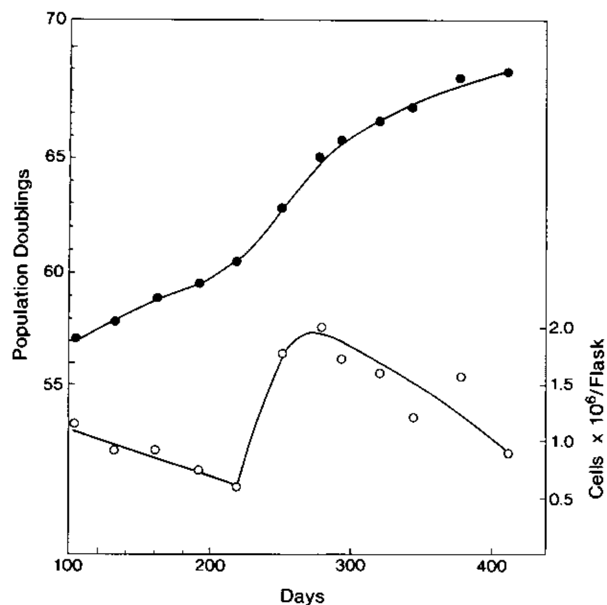

FIG. 7. The cumulative growth of MRC-5 cells in DMEM containing 30 m*M* carnosine in PDs (●) and the yield of cells per flask (○). The cells were transferred from DMEM at PD 55.1, and growth ceased after 413 days (see Table 2).

cortisone [20, 21], ionizing radiation [22, 23], and antisense oligonucleotides to p53 and Rb tumor suppressor mRNAs [24].

The senescent phenotype of Phase III cells is complex, and Hayflick [25] has listed over 100 physiological, biochemical, or genetic parameters which are altered in these cells in comparison to young fibroblasts. This suggests that whatever the primary causes of senescence may be, the end result is a loss of cellular homeostasis which has many effects on the phenotype. This indicates that carnosine may have a role in maintaining homeostasis. One possibility is that free radical damage is responsible for the senescent phenotype and the antioxidant activity of carnosine [3–5] delays or prevents this damage. Another is that carnosine chelates Fe^{2+} ions which might otherwise generate $OH^{\bullet}$ radicals by the Fenton reaction. However, on chemical grounds D-carnosine, as well as homocarnosine and anserine, should be as effective as L-carnosine, provided it is taken up by the cells. The fact that only L-carnosine has specific effects suggests that its metabolism is an important component of its properties.

In collaborative biochemical studies [7, 26], which will be published in detail elsewhere [8], it has been observed

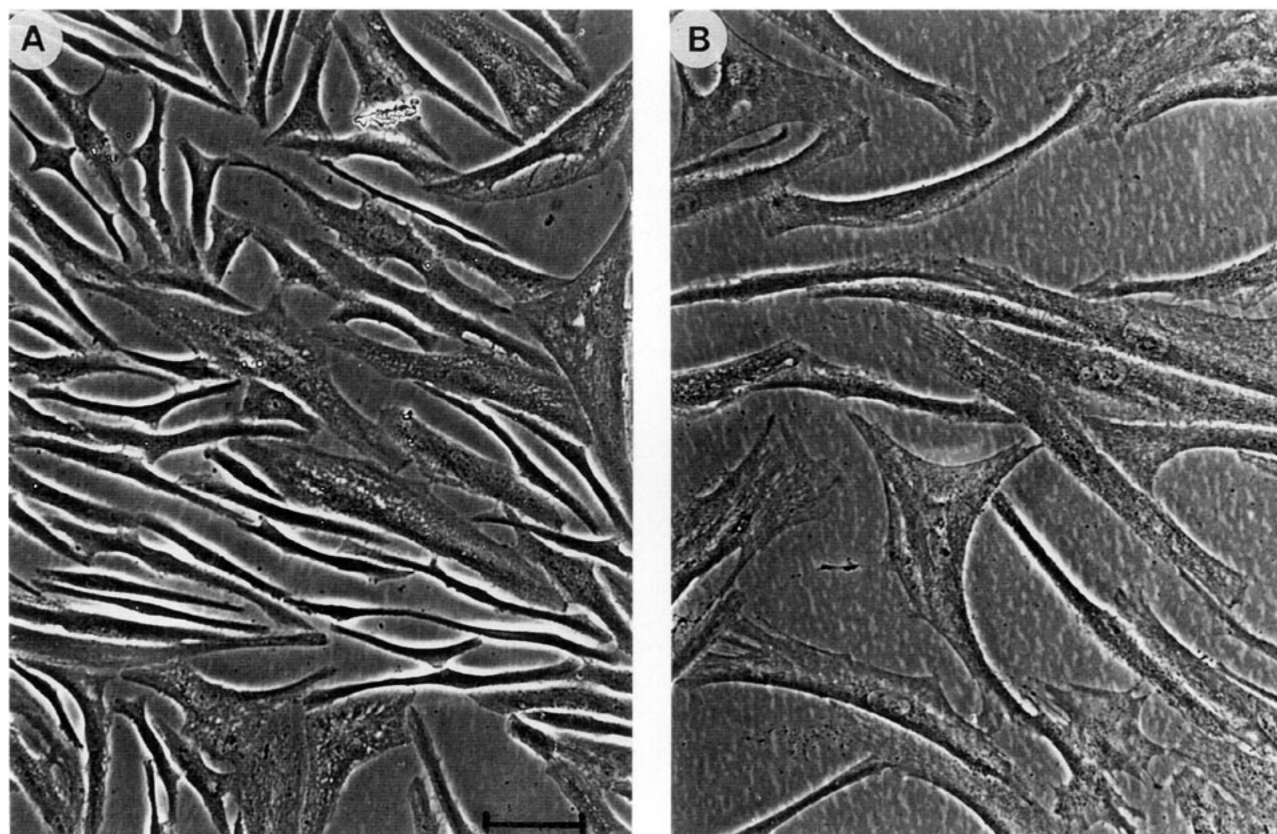

FIG. 8. HFF-1 cells grown in DMEM containing 50 m*M* carnosine. (A) Late passage cells which are subconfluent. These cells eventually became confluent and were subcultured. (B) The final culture which did not become confluent. Bar, 100 μm.

that carnosine can prevent the nonenzymatic glycosylation of proteins. The terminal amino group of the lysine side chains of protein can react with the aldehyde of a linear sugar molecule to produce first a Schiff base and subsequently the Amadori rearrangement forms a carbonyl linkage. The β-amino group of carnosine reacts much more rapidly with a sugar aldehyde than does lysine, so carnosine could prevent the reaction of protein lysine with sugars *in vivo*. There is much evidence that abnormal proteins accumulate in aging cells [27, 28, 29], and one possibility is that advanced glycation end products (AGEs) contribute significantly to the senescent phenotype [30, 31]. If this is so, then carnosine may rejuvenate human fibroblasts by inhibiting glycosylation of proteins. Abnormal proteins in cells are recognized and removed by proteases [27], so the accumulation of defective molecules may be the result of a decrease in proteolytic activity. It is therefore possible that carnosine has significant effects either on reducing the formation of abnormal molecules or on stimulating their removal. Preliminary evidence indicates that protein turnover is significantly altered in cells grown in carnosine [26].

In summary, we suggest that carnosine may have two different effects on human diploid fibroblasts *in vitro*. First, it prevents the onset of the senescent cell morphology and can rejuvenate cells which have become senescent. Second, it affects earlier events during the serial subculture of fibroblasts and this can result in the formation of long-lived clones. In both cases, the Hayflick limit to cell division is nevertheless seen, but the major characteristics of the senescence in control cultures are substantially reduced by carnosine. Our results raise many questions that cannot be answered without further experimentation on the cellular and biochemical effects of carnosine.

Geoffrey Grigg initiated the project to study the effects of carnosine on aging, and we thank him for his continuing encouragement. We also thank Alan Hipkiss for many discussions on the possible biochemical properties of carnosine and Louise Lockley for her assistance in preparing the photographs. Fred Widmer, Peptide Technology Ltd., kindly provided the D-carnosine and Roger Reddel ampoules of HFF-1.

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