and the functions of the genome (*18*). Moreover, arginyl–transfer RNA synthetase (E.C. 6.1.1.19) reacts with (charges) L-canavanine (*19*), which is incorporated into proteins (*20*). It has been suggested that the erroneous protein affects synthesis of messenger RNA molecules and disrupts replication (*5*), and it seems possible that the immune system reacts to abnormal canavanyl proteins by giving rise to the autoantibodies seen in this SLE-like syndrome. Canavanine may also function in enzymatic reactions involving arginine as the preferred substrate (*21*). Finally, an additional mechanism for the analog toxicity may result from the hydrolytic cleavage of L-canavanine by arginase to yield L-canaline, H_2N-O-CH_2-CH_2-CH(NH_2)COOH, and urea. Structurally, L-canaline is an analog of ornithine [H_2N-CH_2-CH_2-CH_2-CH(NH_2)COOH]. Antimetabolic properties may result from L-canaline binding with pyridoxal phosphate and the subsequent inactivation of enzymes that require the B_6 cofactor (*22*).

L-Canavanine is one of the numerous nonprotein amino acids with functional similarities to the components of mammalian proteins (*23*) that occur in higher plants (*24*). Whether they may be ingested and thus induce or reactivate SLE in susceptible humans remains to be explored.

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Calcium and Age in Fibroblasts from Control Subjects and Patients with Cystic Fibrosis

Abstract. *Intracellular calcium increases significantly as human fibroblasts age in culture. The calcium increase occurs 5 to 6 weeks (passages) earlier and is significantly greater in fibroblasts from subjects with cystic fibrosis in comparison with cells from control subjects. Intracellular calcium, which is thought to be a pathogenetic factor in cystic fibrosis, may also be a meaningful marker in cell aging.*

For several decades, sporadic reports of an association between cellular calcium and aging have appeared (*1*). To our knowledge, such a relation has not been described in cultured fibroblasts (*2*), which are used as a model of aging. Because the concentration of intracellular calcium is greater in cells from individuals with cystic fibrosis (CF) (*3*) than in cells from control subjects (*4, 5*) and since cells from individuals with CF appear to age prematurely (*6*), we designed experiments to examine the relation between intracellular calcium and passage number (weekly age in culture) in fibroblasts from control subjects and from patients with CF. We report here results that (i) confirm that increased intracellular calcium occurs in cells from patients with CF in comparison with age- and

Table 1. Description of fibroblast strains used in assays of cellular calcium by atomic absorption.

Fibroblast strain number	Sex	Age (years)	Passage at which calcium was assayed	
			Experiment 1	Experiment 2
		Cells from patients with CF		
29	F	12	15	
44	M	10	15	
45	F	7	15	
46	M	16	6, 7, 15	
55	F	24	15	5 to 20
63	F	(3 months)		4 to 20
69	M	17	6, 7	5 to 20
70	M	21	6, 7	3 to 20
71	M	7	6, 7	5 to 7
72	F	20	6, 7	3 to 20
		Cells from control subjects		
31	F	13	15	
32	M	13	15	
57	F	25	15	
59	M	13	15	
61	F	8	15	
82	F	30		4 to 20
83	F	22	6, 7	5 to 6
84	M	20	6, 7	
85	M	6	6, 7	3 to 20
90	M	20	6, 7	3 to 20
91	M	20	6, 7	4 to 6
92	F	(3½ months)		3 to 20
93	F	(5 months)		
94	F	(5 months)		

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sex-matched controls (*5*), (ii) demonstrate that intracellular calcium increases in cells from both CF patients and controls as the cells age in vitro, and (iii) reveal a significantly later and less profound calcium increase in control cells compared to cells from patients with CF.

Cultured skin fibroblasts were obtained, maintained, frozen, thawed, and used as described previously (*7*). Monolayers were established in 75-cm^2 tissue culture flasks (Falcon) and grown to confluence. Cells were removed with trypsin and EDTA (1:250 by volume; Gibco) and resuspended in Hanks balanced salt solution (Gibco), and cells from a portion of the suspension were counted in a ZBI Coulter counter. The remaining cells were washed three times with 0.25 percent lanthanum chloride (Fisher) in 0.9 percent NaCl to remove membrane-bound calcium and to prevent efflux of calcium from the cells (*8*). We used lanthanum chloride (0.25 percent) dissolved in distilled H_2O to suspend the cells. The suspensions were transferred to crucibles that had been treated with acid (3*N* HCl) and then washed (double-distilled deionized water). The mixtures were dried on a hot plate and the cells heated overnight at 600°C. The ash was dissolved in 1 ml of 0.2*N* HCl and dried on a hot plate and redissolved in 1 ml of distilled H_2O. The calcium content of cells from all control subjects and CF patients was determined in duplicate with a Jarrell-Ash (model 82-500 MVAA) atomic absorption spectrophotometer. Cells from control subjects and patients with CF were always assayed on the same day.

Intracellular calcium was determined at passages 6 and 7 on cells from five patients with CF and five age- and sex-matched control subjects, and at passage 15 on a different set of five matched pairs (Table 1, experiment 1). The mean calcium concentration (expressed as 10^{-7} μg of calcium per cell) was significantly higher in CF fibroblasts in comparison with controls at each passage [passages 6 and 7: CF, 16.7 ± 1.7; control, 8.7 ± 0.7 ($P < .005$); passage 15: CF, 54.3 ± 8.1; control, 20.9 ± 3.4 ($P < .005$), unpaired Student's *t*-test]. In both groups, calcium concentration was greater at passage 15 than at earlier passages ($P < .005$). We tested whether the units in which calcium was expressed had an effect on our conclusions about the differences between cells from control subjects and patients with CF. The calcium content of cells from CF patients was greater than that of controls when expressed in terms of flask, cell number, milligrams of protein, or milligrams of ash. We learned also that calcium yield as assayed by atomic absorption was two to three times

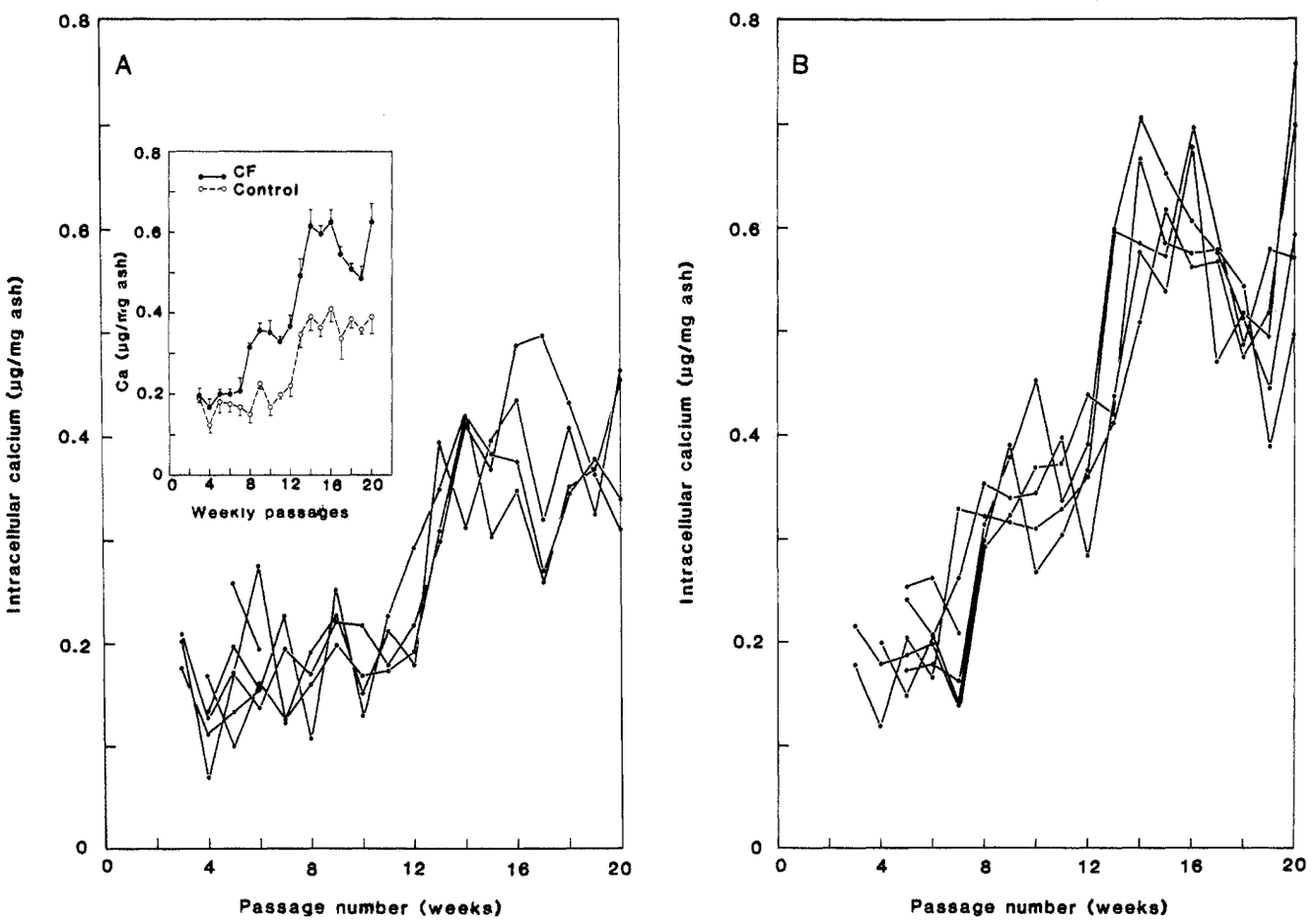

Fig. 1. Intracellular calcium in fibroblasts from (A) control subjects and (B) patients with CF, determined by atomic absorption spectrophotometry. Each point (●) represents the mean of duplicate determinations. Each line on the graph represents the cells from a different subject assayed weekly. At each passage cells from all the control subjects and all the CF patients were assayed on the same day. The cells were seeded in 100-mm culture dishes (Falcon) with 10 percent fetal calf serum (Gibco) in minimum essential medium (Gibco), and incubated at 37°C with 95 percent air and 5 percent CO_2 for 7 days. The medium was removed and monolayers were washed three times with 0.25 percent lanthanum chloride (Fisher) in 0.9 percent NaCl. The cells were scraped from monolayers in the presence of 3 ml of 0.25 percent lanthanum chloride. The suspension was dried and ashed in acid-treated crucibles overnight at 600°C. Ash weight was determined. Calcium was analyzed in an atomic absorption spectrophotometer (Varian AA 475) (*10*). (Inset) Means ± standard error of values in (A) and (B); $P < .25$ at passages 4 through 8 and $P < .01$ at passages 9 through 20 (Student's *t*-test).

greater when cells were removed from monolayers by scraping as opposed to removal with trypsin-EDTA. Accordingly, in subsequent experiments we harvested the fibroblasts by scraping and for convenience expressed cell calcium in terms of milligrams of cell ash.

Because of the difference in the calcium content of cells from control subjects and CF patients between early and late passages, we decided to examine systematically the intracellular calcium content relative to passage number. Passage 3 was the earliest at which we could obtain a sufficient number of cells for calcium analyses. We first assayed calcium in lymphocytes from CF patients and controls (*9*) in order to rule out a differential effect of culture in vitro on the two genotypes. The cells were obtained (*10*) and prepared for atomic absorption in the same way as the fibroblasts. The mean calcium concentration in lymphocytes from patients with CF ($N = 10$) was $10^{-7} \times 4.1 \pm 0.4$ μg per cell, that is, more than twice that in lymphocytes from control patients ($N = 6$): $10^{-7} \times 1.8 \pm 0.2$ μg per cell ($P < .005$). These results, and data with other cell types (*4, 5, 11*), suggest that increased intracellular calcium is indeed characteristic of cells from individuals with CF and that the increase is not secondary to an artifact in vitro.

We thawed fibroblasts from six patients with CF and six age- and sex-matched control subjects (Table 1, experiment 2) in order to assay cellular calcium by atomic absorption each week for several months. The cell cultures from one CF patient and two control subjects were terminated after the seventh and sixth passage, respectively, because of evidence of microbial contamination. Data for the cells from these three subjects, until their termination, are included in Fig. 1. From passages 4 through 7, the mean calcium concentration was not statistically significantly greater in CF cells ($P < .25$ at each passage). Thereafter, from passages 8 through 20, the calcium concentration in CF cells was consistently and highly significantly greater than in controls [$P < .25$ to $P < .0005$ at different passages (inset in Fig. 1)]. No significant increase in calcium concentration in control cells occurred from passages 3 through 12; between passages 12 and 13, the calcium concentration in the control strains increased significantly ($P < .01$) after which it remained at a plateau. No statistically significant change in calcium concentration in CF strains occurred between passages 3 and 7. The calcium concentration in the CF strains increased significantly between passages 7 and 14 ($P < .0005$), after which a plateau was reached (*12*). When cellular calcium in both groups reached a plateau (passages 14 to 20), the mean calcium concentration was 50 percent greater in CF (0.57 ± 0.02 μg of calcium per milligram of ash, $N = 5$) than in control (0.38 ± 0.01 μg of calcium per milligram of ash, $N = 4$) strains ($P < .0005$) (*13*). These experiments did not enable us to distinguish, as the cells aged, between increased calcium in all or most cells of the monolayer or an increase in the proportion of cells with a higher cellular calcium content. Nevertheless, a comparable process seemed to occur in both the control and CF cells, but the initial plateau ended much earlier in the CF cells, with a sharp increase in calcium occurring five or six passages (weeks) earlier than in control cells. Similar results were obtained when we used different experimental methods [cells being harvested with trypsin and EDTA (experiment 1) or by scraping (experiment 2)] and expressed the results in different ways (calcium per cell or calcium per milligram of ash). In experiment 1, the mean cellular calcium was 3.25 and 2.40 times greater at passage 15 than at passages 6 and 7 in the CF and control cells, respectively. In experiment 2, the mean cellular calcium was 2.92 and 2.09 times greater at passage 15 than at passages 6 and 7 in the CF and control cells, respectively.

Significant variability in strains from day to day and from strain to strain is the rule in tissue culture experiments. The similarity of the calcium content of cells of the same genotype (CF or control) and over passages is, therefore, noteworthy. Several interpretations of the curves in Fig. 1 are possible. Clearly, calcium in the cells from patients with CF was greater than in cells from matched control subjects. Similarly, the calcium content was consistently greater in the older cells. The data for cells in which calcium was assayed at passages 3 through 20 suggest that in comparison with controls the CF curves are shifted to the left. It appears that the increase in intracellular calcium after passage 12 in control cells is similar to that which occurs in CF cells at passage 6. Where the calcium concentration in the control strains remains at this plateau, the concentration in CF cells appears to rise to a higher plateau after passage 12 (*12*).

The potential role of intracellular calcium in the pathogenesis of CF has been discussed elsewhere (*4*) as has the possibility of premature aging in CF (*6*). The relation between aging and cellular calcium in cultured mammalian cells has not been noted previously. It is unclear whether the change in calcium is a fundamental trigger in cell aging or is an expression of some other aspect of senescence. In either case, the change appears to be sufficiently consistent and dramatic to serve as a useful marker in aging studies.

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12. We do not understand the apparent decrease in calcium in CF strains at passages 17 to 19 (Fig. 1B). These lower values were still significantly greater than those for control strains assayed at the same time. Since a parallel decrease did not occur at these passages in the control strains, an experimental weekly effect may be ruled out. The mean calcium concentrations in CF strains at 20 weeks returned to those at 14 to 16 weeks. We assume, therefore, that intracellular calcium in CF strains reaches a plateau after 14 weeks in culture.
13. The mean value of cell calcium for each strain from passages 14 through 20 was determined. Means ± standard error were calculated for the five CF and four control strains that survived 20 weekly passages.
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