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8. In the in vitro assay for PTTH, one PG of a pair in an animal serves as the experimental gland and is incubated in medium containing a portion of test sample while the contralateral control gland is incubated in medium alone. The assay is performed in Grace's medium, and the amount of medium varies depending on experimental design. It is never less than 20 percent by volume to ensure that the gland is capable of being activated maximally by PTTH. Activation is expressed as an activation ratio (A_r), which is the ecdysone synthesized and released by the experimental gland in 2 hours divided by that released by the control gland. Ecdysone synthesis is quantified by an ecdysone radioimmunoassay (RIA) in which a sample (0.01 ml) of the incubation medium (0.025 ml) is assayed directly. A macro RIA with an ecdysone standard concentration range of 0.25 to 32 ng was used (*11*).
9. The in vitro PG assay was used to quantify units of PTTH activity. A standard PTTH unit is the amount of neurohormone present in a day 1 pupal brain of *Manduca*. The value of the standard PTTH unit was determined from the dose of day 1 pupal brain extract (ED_{50}) needed to half-maximally activate the PG (A_{50}) in a dose-response curve of PG activation in vitro. For day 1 pupal brains, a mean ED_{50} of 0.06 brain equivalents elicited the A_{50}; thus with one brain, 16.7 (the reciprocal of 0.06) in vitro PG assays can be run at the A_{50}. One PTTH unit is therefore the amount of neurohormone that can generate 16.7 in vitro assays at the A_{50}. The PTTH activity in one PTTH unit represents the combined activity of big and small PTTH. Because of the different relative amounts of these two neurohormones in the brain, the different efficiencies with which they are purified, and the stage-specific differences in the PG response they elicit, the PTTH unit had to be redefined for big and small PTTH once they were separated. The definition of these units retained as its basis the ED_{50} value derived from a dose-response curve of PG activation in vitro with each neurohormone. Thus one unit of big PTTH is the amount of this hormone that can generate 16.7 in vitro assays at the A_{50} when either day 0 pupal or day 3 larval PG are used. Similarly, one unit of small PTTH is the amount of this hormone that can generate 16.7 in vitro assays at the A_{50} when day 3 larval PG are used. Thus the ED_{50} in units of big and small PTTH in the in vitro assay is 0.06 as it is with a standard PTTH unit. To quantify PTTH units in a sample requires the generation of a dose-response curve of PG activation; from this curve the number of in vitro assays that can be run with the sample at the A_{50} is determined. This number is divided by 16.7 to yield total PTTH units.
10. After heat treatment and the quantification of PTTH content, the brain extract was subjected to ultrafiltration and diafiltration with distilled water (Amicon YM-2). The retentate, free of small molecules (< 1 kD), was assayed for PTTH to ensure minimum loss of activity and then lyophilized and stored at −20°C.
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14. Big and small PTTH (12 to 14 units from same stocks as in Fig. 2) were incubated individually with 1.45 units of protease (Sigma) and 4.12 units of trypsin (Boehringer Mannheim) in tris buffer (0.046*M*, *p*H 8.1) for 4 hours at 25°C. The loss of PTTH activity was due to proteolytic hydrolysis since heat-denatured or inhibitor-inactivated enzyme had no effect on the ability of nonhydrolyzed PTTH to activate the PG.
15. Analytical isoelectric focusing was performed (LKB Instruments, application note 250). Fractions were eluted with 0.2 ml of phosphate-buffered saline (0.01*M* NaH_2PO_4, 0.15*M* NaCl, *p*H 7.2), mixed with Grace's medium 4:1 (by volume), respectively; the *p*H was adjusted to 6.8 and PTTH activity was assayed. The *p*I for big PTTH was determined on a 4 to 6.5 *p*H gradient and the *p*I for small PTTH was determined on 4 to 6.5 (*p*I 5.4) and 3.5 to 9.5 (*p*I 3.7) *p*H gradients. Ampholines were not removed before assay since at *p*H 6.8 they did not have an adverse effect on the ability of the PG to be activated by PTTH.
16. D. Gibbs and L. M. Riddiford, *J. Exp. Biol.* **66**, 255 (1977).
17. L. M. Riddiford, *Nature (London)* **256**, 115 (1976). Pupal commitment: early in the last larval instar a small increase in the hemolymph ecdysteroid titer occurs in response to PTTH activation of the PG, and this increase in the absence of juvenile hormone covertly commits the larval tissues to become pupal. The expression of this commitment occurs later in the instar in response to a second, dramatic increase in the ecdysteroid titer.
18. H. Ishizaki reported (at the Second International CNRS Symposium on the Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones in Strasbourg, France, September 1983) that adult heads of *Bombyx mori* contain a 22-kD peptide having PTTH activity in *Bombyx*. This is in addition to the 4.4-kD moiety which is not active in the *Bombyx* assay but is active in a *Philosamia cynthia ricini* assay.
19. We thank N. Granger for editorial comments and J. Hobgood and M. Crotteau for rearing the more than 100,000 *Manduca* needed for this study. Supported by NIH grants NS-18791, AM-31642 (W.E.B.) and AM-30118 (L.I.G.), NSF grant PCM-8116931 (L.I.G.) and USDA grant 59-2176 (W.E.B.).
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Reinitiation of Growth in Senescent Mouse Mammary Epithelium in Response to Cholera Toxin

Abstract. *Several lines of mouse mammary tissue that had been serially transplanted until mitotic senescence was reached were exposed in vivo to plastic implants that slowly released cholera toxin. Gland tissue surrounding the implants displayed new end buds, indicating reinitiation of growth and morphogenesis. The ability of cholera toxin, which elevates intracellular adenosine 3',5'-monophosphate, to temporarily reverse the senescent phenotype suggests that this mitotic dysfunction results not from generalized cellular deterioration but from specific changes in cell regulation.*

Normal cells consistently display a limited capacity for proliferation when serially transferred in culture (*1, 2*) or when passaged in young, isogeneic hosts in vivo (*3*). An example of this phenomenon, often interpreted as an expression of cellular aging, is found in mouse mammary epithelium. Young mammary ducts exhibit vigorous growth when transplanted into gland-free mammary fat pads, but undergo a progressive reduction in growth rate with serial transplantation (*4*), even though the transplanted tissue maintains its normal stromal associations (*5*) and is propagated in a hormonal and nutritional environment that is optimal for continuous proliferation and morphogenesis. This loss of replicative potential by normal cells appears irreversible, and immortal transplant lines can be obtained only by transformation into preneoplastic (hyperplastic) or neoplastic cell types (*6*).

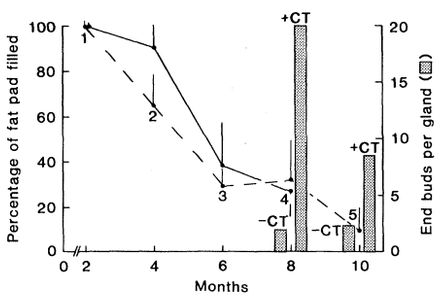

Fig. 1. Growth of mammary ducts in two of the tissue lines used for experiments represented in Fig. 2 and Table 1 (solid line, experiment 1; dashed line, experiment 3). Each transplant line was initiated by tissue taken from a 3-month-old BALB/c donor. End-bud number, a measure of growth rate, is indicated by bars at generations 4 and 5 for experiments 1 and 3, respectively. Vertical lines are 95 percent confidence intervals. *CT*, cholera toxin.

Agents that raise the intracellular concentration of adenosine 3',5'-monophosphate (cyclic AMP), such as cholera toxin (*7*), are mitogenic in vitro for a variety of epithelial cells (*8–11*); mammary cells are stimulated to increase DNA synthesis and to divide in both monolayer (*12*) and collagen gel cultures (*13*). In vivo, increased cyclic AMP in mammary tissue is correlated with pregnancy (*14*) and with several breast carcinomas (*15*). In normal mammary ductal tissue cyclic AMP is a positive growth regulator, as shown by administration of cholera toxin and other cyclic AMP–active agents to local areas of mammary glands in ovariectomized mice (*16*). Small plastic implants (*17, 18*) were used to gradually release the agents. Cholera toxin stimulated the appearance of new end buds, which grew rapidly and participated in normal ductal morphogenesis. In young intact animals, cyclic AMP–active agents did not increase the elongation rate of mammary ducts, which were already growing at the maximum rate.

We undertook experiments to determine whether serially aged, growth-retarded mammary duct cells retain the ability to respond to cyclic AMP–active agents. Each of three mammary transplant lines was initiated by transplanting fragments of mammary ducts from a 3-

Table 1. Stimulation of serially aged mammary duct tissue by cholera toxin. Data for experiments 1 through 5 are for three transplant lines. Each of the fourth pair of glands, which had been cleared of host mammary glands (*17*), contained transplanted tissue. One gland was treated with a cholera toxin implant, while the contralateral gland received a blank implant. Duration of treatment and dosage were as follows: in experiment 1, 5 days and 0.2 μg; in experiment 2, 7 days and 0.5 μg; and in experiments 3 to 5, 5 days and 0.4 μg. Aged tissue from the same transplant lines but from animals receiving no implants was also analyzed (experiments 6 to 8). N.A., not applicable (number of end buds insufficient for significant counts).

Experiment	Generation	Implant	Number of glands	End buds per gland	End-bud cells per gland†	Labeled end-bud cells per gland†,‡
1	4	Cholera toxin	7	20.6*	6,547*	1,237*
		Contralateral blank	7	1.9	238	36
2	4	Cholera toxin	5	8.2**	2,383*	385*
		Contralateral blank	5	3.6	819	180
3	5	Cholera toxin	10	8.6*	2,918*	779*
		Contralateral blank	10	2.3	641	138
4	5	Subunit A	7	0.86	488	89
		Contralateral blank	9	0.11	40	4
5	5	Subunit B	5	2.6	938	184
		Contralateral blank	5	1.0	282	31
6	1	None	12	29	27,127	5,549
7	4	None	11	0.30	N.A.	N.A.
8	5	None	6	0.17	N.A.	N.A.

*Significantly different from corresponding control value [$\chi^2(1) = 10.83$, $P < 0.001$]. **$P < 0.01$. †Counts are based on three longitudinal sections centered on the lumen and represent only a fraction of the total cells present. ‡A single injection of [^{3}H]thymidine (100 μCi; 79 Ci/mmole) was given to each animal 45 minutes before it was killed.

month-old BALB/cCrl female into a group of 3-month-old isogeneic females whose fourth pair of mammary fat pads had been cleared of host glands (*19*). Transplantation into both of these gland-free fat pads in each host was continued at 2-month intervals (*4*), with the most vigorous outgrowths being used for serial propagation. By the fourth transfer generation the average growth rate, measured by the percentage of fat pad filled within the 2-month period of outgrowth, was reduced to 25 percent or less of the rate for generation 1 (Fig. 1).

Since end buds represent the growth points of mammary ducts, the rate of expansion of a mammary tree is a function of the number of end buds in a gland and the growth rate of individual buds. Generation 1 outgrowths had large end buds, indicating rapid growth, whereas in serially aged outgrowths end buds were reduced or absent and the terminal ducts were often spindly (Fig. 2b). When plastic implants containing cholera toxin were placed in fat pads carrying serially aged ductal tissue, new end buds appeared at ductal tips in the region of the implants within 5 days (Fig. 2a), with treated glands showing as much as a tenfold increase in end buds (Fig. 1). These end buds were normal in size and structure, displayed high levels of DNA synthetic activity (Fig. 2, c to e), and contained numerous mitotic figures. To-

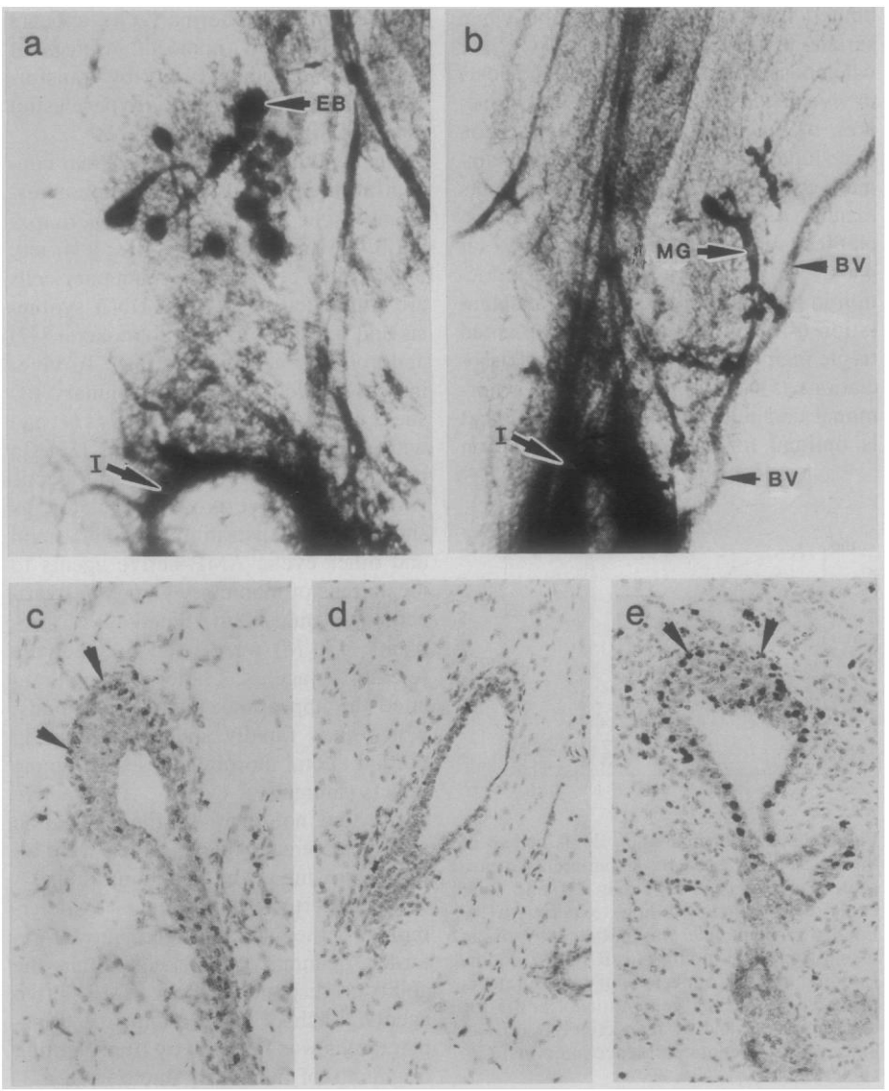

Fig. 2. Stimulation of serially aged mammary outgrowths by cholera toxin. (a) Whole mount of generation 5 outgrowth filling 5 percent of the available fat pad. Six weeks after transplantation the gland was exposed to an Elvax implant (*I*) containing 4 μg of cholera toxin. Well-developed end buds (*EB*) are found at the tip of each duct, and their general form, size, and growth pattern appear normal (×37). (b) Control gland whole mount showing generation 5 outgrowth (filling 5 percent of the fat pad) from the contralateral side of the same host as in (a). The Elvax implant contained no cholera toxin; the gland lacks end buds and is comparatively unstimulated. *BV*, blood vessel; *MG*, mammary gland. (×37). (c to e) DNA autoradiography of mammary end buds (×287). Animals received a single injection of [^{3}H]thymidine (100 μCi; 79 Ci/mmole; New England Nuclear) 45 minutes before being killed. (c) End bud from normal 5-week-old mouse, showing typical growth in young tissue. Most of the cells in the outer layer are labeled; these cap cells (arrowheads) represent a stem cell population that is conspicuous in rapidly growing end buds. (d) Untreated end bud from generation 4 transplant line. The body is elongate and thin-walled. One labeled epithelial cell appears in this section, and the cap cell layer is not seen. (e) Generation 4 end bud after 5 days of exposure to an Elvax implant containing 3.0 μg of cholera toxin. The bulbous shape and heavy labeling in cap cells (arrows) make it indistinguishable from a young gland (c).

tal cell numbers were substantially increased as well, indicating cell division (Table 1).

A direct effect of cholera toxin is indicated by the larger number of end buds in treated glands than in contralateral glands that received blank implants (Fig. 1 and Table 1). A slight systemic effect is also apparent if transplanted glands from untreated mice (mice without implants) are compared with glands from animals carrying a cholera toxin implant (Table 1). The specificity of the effect is indicated by the finding that the intact cholera toxin molecule had a substantially greater influence than its A or B subunits administered separately (Table 1).

Although systemically administered cholera toxin results in hyperplastic or dysplastic responses in skin (*20*), abnormalities were not observed in cholera toxin–treated senescent mammary tissue. After 10 days of cholera toxin stimulation the implant's effects were largely exhausted (*16*), the end buds had regressed, and there was no evidence of hyperplasia or other abnormal growth states.

The end buds formed in response to treatment were typically multilayered, with a distinct layer of undifferentiated cap cells (Fig. 2e), the putative stem cell population from which myoepithelial and perhaps other mammary cell types are derived (*21*). The reappearance of stem cells and the normal morphology of the end buds indicate that the tight coupling between cell proliferation and branching morphogenesis, characteristic of young tissue, remains intact.

In vitro, noncycling cells in certain lines of human fibroblasts have been stimulated to enter DNA synthesis in response to supplementation of the medium with cortisol (*22*). This response was possible, according to one interpretation, only because the cells tested had been propagated in medium that was hormonally suboptimal. Other reported cases of reinitiation of DNA synthesis in nuclei of senescent human fibroblasts involved fusion with HeLa or SV-40 transformed cells (*23*) or infection of senescent cells with DNA tumor viruses (*24*). Our results suggest that serially aged tissue can be restored, at least during treatment, to normal levels of growth by an agent that is not oncogenic and whose mode of action is expressed on well-studied metabolic pathways. Because we used whole glands we do not know whether the primary action of cholera toxin is on the epithelial or the stromal components.

Earlier investigations showed that growth-exhausted cells in mammary tissue passaged for long periods appear morphologically normal rather than exhibiting the generalized degenerative changes characteristic of senescent cells in culture. We have also reported that aged mammary ductal cells are capable of responding to the hormones of pregnancy by proliferating to the stage of alveolar differentiation, again indicating that major cell functions remain intact (*25*). The ability of cholera toxin to stimulate DNA synthesis in serially aged mammary cells further supports the interpretation that mitotic senescence is associated with age-related alterations in one or more regulatory pathways, which, in the case of mammary cells, may be regulated by cyclic AMP. The present results provide a temporarily "reversible" aging system that will be useful for the study of these interactions.

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Dietary Restriction Retards Age-Related Loss of Gamma Crystallins in the Mouse Lens

Abstract. *The soluble crystallins in lenses from diet-restricted and control mice of diverse ages (2, 11, or 30 months) were studied by high-performance liquid chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Results obtained with both methods suggest that dietary restriction decelerates age-related loss of soluble gamma crystallins.*

Dietary restriction, when started early in life and continued until death, increases average and maximal survival times of mice (*1*) and rats (*2*). It extends maximal survival times more reliably than other antiaging strategies tested in rodents (*3*) and is the only procedure yet tested in homeotherms that slows age-related increases in mortality rates (*4*). Longer life-spans for rodents on restricted diets are associated with a lower incidence and later onset of several late-life diseases (*5*). Rodents on diet restriction show more youthful physiologic (*6*) and immunologic (*7*) responses than do age-matched controls on unrestricted diets. The antiaging action of dietary restriction appears to depend on restriction of calories (a 25 to 50 percent decrease in most studies) without a decrease in essential nutrients (undernutrition without malnutrition).

For many years the eye lens has been a model for studying age-related biochemical changes in structural proteins and enzymes (*8, 9*). The lens of the vertebrate eye is composed primarily of concentric layers of long hexagonal units called lens fiber cells. These fiber cells differentiate from an epithelial monolayer that covers the anterior surface of the lens and terminates at the equator. During the process of differentiation (which occurs throughout life), the equatorial epithelial cells elongate and follow a curved course that extends from the anterior to the posterior pole of the lens. New lens fibers are deposited over the older ones, the latter being compressed toward the interior of the region known