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# Circadian Stage-Dependent Effects of Epidermal Growth Factor on Deoxyribonucleic Acid Synthesis in Ten Different Organs of the Adult Male Mouse*

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**ABSTRACT.** Epidermal growth factor (EGF) previously isolated from the submandibular gland of mice was injected ip at different circadian stages into separate subgroups of adult male $CD2F_1$ mice. Subsequent to each of the five time points of injection (0900, 1500, 1800, 2100, and 0300 h for animals standardized to 12 h of light alternating with 12 h of darkness: light, 0600–1800 h; dark, 1800–0600 h), five animals were killed at 4, 8, and 12 h after the EGF injection; comparable control groups were injected only with the carrier substance. Thirty minutes before sacrifice, each mouse was injected ip with 24 μCi [$^3$H]thymidine. Incorporation of [$^3$H]thymidine into the DNA of the aorta, lung, liver, cornea, testes, kidney, parotid, thymus, spleen, and bone marrow as well as the mitotic index of the corneal epithelium was determined. The results indicate that EGF may play a role in the positive control of growth of many of these tissues, especially the aorta, lung, liver, and cornea. EGF may also play a role in inhibiting growth of the thymus, spleen, and bone marrow. Moreover, the stimulatory effect of EGF on the growth of the various tissues appears to be especially enhanced in mice injected at 1500 h and killed 4 h later at 1900 h. (*Endocrinology* **109:** 644, 1981)

EPIDERMAL growth factor (EGF) is a polypeptide hormone that has been isolated from the submandibular gland of the adult mouse. EGF stimulates cell division *in vitro* (1, 2) and in the neonatal mouse (3), promotes lung maturation in the fetal rabbit (4), and stimulates hepatic DNA synthesis in the adult rat (5); however, its effect on DNA synthesis in different tissues of the adult mouse has not been adequately studied. We have recently demonstrated that EGF potently stimulates DNA synthesis from the tongue to the rectum of the digestive tract, leading us to propose that EGF plays a major role in the control of gastrointestinal growth (6, 7).

In this study, using tissues of mice from which our previous results derive, we have characterized the growth response of additional tissues to EGF. The effect of EGF on DNA synthesis was examined in the following tissues: aorta, cornea, kidney, liver, lung, parotid, testes, thymus, bone marrow, and spleen. Also, the effect of EGF on the mitotic index of the corneal epithelium was determined.

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## Materials and Methods

Six-week-old male $CD2F_1$ mice were received from Laboratory Supply Co. (Indianapolis, IN) on April 25, 1978. The animals were subdivided into groups of five per cage, and four or five such cages were placed in sound-attenuated, temperature-regulated isolation chambers. Each chamber was illuminated from 0600–1800 h daily, with food and water available *ad libitum*. The mice were maintained in this manner for 2 weeks before the experiment, although the cages were replaced with clean ones once each week.

The EGF was isolated from the submandibular gland of adult male mice by the method of Savage and Cohen (8). One day before the experiment, EGF was dissolved in 0.9% NaCl solution containing 0.05% bovine serum albumin, aseptically distributed into five sterile vials and frozen until used. EGF yielded a single band on polyacrylamide gel electrophoresis at pH 8.5. Crystalline bovine serum albumin was obtained from the Sigma Chemical Co. (St. Louis, MO). The globulin content was less than 0.1%. No mitogenic activity was found. The carrier without the EGF was treated similarly and served as the control substance.

At 1500 h on May 14, 1978, a group of 30 mice, consisting of 15 experimental and 15 control mice, was injected three times ip with 0.1 ml of either the EGF solution or the vehicle alone. The EGF was given in a dose of 8.33 μg/injection or a total of 25 μg/mouse. The three equally spaced injections for each mouse spanned 90 min (*i.e.* 1415, 1500, and 1545 h). This procedure was repeated on similar groups of mice at 1800, 2100, 0300, and 0900 h. From each of the five groups injected, three subgroups of five experimental and five control mice were killed

4, 8, and 12 h after the initial injection. During the dark phase, all injections were done under a dim red light, and the injected mice were immediately returned to the dark environment.

Thirty minutes before the animals were killed, each mouse was injected ip with 24 μCi [$^3$H]thymidine (17 Ci/mmol; New England Nuclear Corp., Boston, MA). Mice were killed by rapid cervical dislocation, the thoracoabdominal cavity was opened, and the animals were fixed in a 10% buffered formalin solution. After fixation of the carcass for 2 weeks, pieces of the studied tissues were removed. The DNA was then extracted from each piece of tissue by the method of Ogur and Rosen (9), with the modification that the RNA hydrolysis was carried out in a 1 N NaOH solution at 60 C for 18 h. Details of this modified procedure have been published (10). The mitotic index in the cornea was determined as previously described (11).

A one-way analysis of variance was used to test for the statistical significance among the different time points in DNA synthesis for each set of time-series data of both experimental and control animals. Student's $t$ test was used to compare differences in DNA synthesis between experimental and control data at specific hours.

## Results

### *Four hours after injection*

Whenever the stimulation or inhibition of DNA synthesis in a particular tissue by EGF is referred to, it can be assumed that the difference between the mean absolute values in DNA synthesis between the experimental and control groups was found to be statistically significant ($P < 0.05$) unless indicated otherwise by "NS". Nonstatistically significant values (NS) are presented in the text and in Table 1 only when borderline statistical significance was obtained ($P > 0.05$ but $\leq 0.10$) because statistical significance might have been obtained had the experimental and control groups contained more than five animals each. Precise $P$ values for all the sets of data cited in this paper are given in Table 1.

Epidermal growth factor stimulated DNA synthesis as early as 4 h after injection. At 4 h after injection, EGF stimulated DNA synthesis three out of the five times in the cornea (0900/1300 h, 1500/1900 h, 1800/2200 h; the first time indicates the injection time and the second, the killing time) and also in three out of the five times in the spleen (1500/1900 h, NS; 1800/2200 h, NS; 0300/0700 h). EGF stimulated DNA synthesis two out of the five times in the testes (0900/1300 h; 1500/1900 h, NS), and thymus (1500/1900 h; 2100/0100 h, NS), and finally, one out of the five times in the aorta (whole abdominal aorta was used but the site of labeling was not localized), kidney, liver, lung, and parotid (1500/1900 h for all tissues). Thus, EGF stimulated DNA synthesis at 4 h after the 1500 h injection in all of the examined tissues, except for the bone marrow. Moreover, EGF stimulated the mitotic index of the corneal epithelium twice (0300/0700 h; 0900/1300 h) (Table 1).

At 4 h after injection, the greatest percent increases in DNA synthesis in the responsive tissues were 415% for the parotid (1500/1900 h), 369% for the cornea (0900/1300 h), 144% for the aorta (1500/1900 h), 98% for the thymus (1500/1900 h), 90% for the testes (0900/1300 h), 47% for the kidney (1500/1900 h), and 38% for the spleen (1800/2200 h, NS). The greatest percent increase in the mitotic index of the corneal epithelium at this time was 250% (0900/1300 h) (Table 1).

EGF inhibited DNA synthesis at 4 h after injection two out of the five times in the cornea (2100/0100 h; 0300/0700 h), testes (1800/2200 h, NS; 2100/0100 h, NS), and bone marrow (1500/1900 h; 0300/0700 h, NS). EGF also inhibited DNA synthesis once in the parotid (0900/1300 h), spleen (0900/1300 h, NS), and thymus (0300/0700 h). Furthermore, EGF inhibited the mitotic index of the corneal epithelium once (2100/0100 h) (Table 1).

The greatest percent decreases in DNA synthesis at 4 h after injection in the affected tissues were 65% for the cornea (0300/0700 h), 45% for the testes (2100/0100 h, NS), 28% for the parotid (0900/1300 h, NS), 25% for the spleen (0900/1300 h, NS), 22% for the thymus (0300/0700 h), and 18% for the bone marrow (1500/1900 h). The greatest percent decrease in the corneal epithelium mitotic index was 37% (2100/0100 h) (Table 1).

### *Eight hours after injection*

At 8 h after injection, EGF stimulated DNA synthesis four out of the five times in the lung (0900/1700 h; 1500/2300 h; 1800/0200 h; 0300/1100 h); three out of the five times in the cornea (1500/2300 h; 1800/0200 h; 0300/1100 h) and in the aorta (0900/1700 h; 1800/0200 h; 0300/1100 h, NS); two out of the five times in the liver (0900/1700 h; 0300/1100 h, NS), in the kidney (0900/1700 h, NS; 1500/2300 h), and in the parotid (1500/2300 h; 1800/0200 h, NS); and finally once in the bone marrow (0900/1700 h, NS) and in the testes (2100/0500 h). EGF also stimulated the mitotic index of the corneal epithelium four out of the five times (1500/2300 h; 1800/0200 h; 2100/0500 h; 0300/1100 h). At no time at 8 h after injection did EGF stimulate DNA synthesis in either the thymus or spleen (Table 1).

The greatest percent increases in DNA synthesis in the affected tissues at 8 h after injection were 157% for the cornea (1800/0200 h), 123% for the testes (2100/0500 h), 92% for the aorta (1800/0200 h), 65% for the parotid (1500/2300 h), 56% for the lung (1800/0200 h), 32% for the liver (0300/1100 h, NS), 29% for the kidney (1500/2300 h), and 14% for the bone marrow (0900/1700 h, NS) (Table 1).

At 8 h after injection, EGF inhibited DNA synthesis three out of the five times in the spleen (1500/2300 h, 2100/0500 h; 0300/1100 h); two out of the five times in

TABLE 1. Percentage increase or decrease from control values of mean levels of incorporation of [$^3$H]thymidine into various tissues of mice injected at different circadian stages with EGF and killed 4, 8 or 12 h later

| Tissue | Time of EGF injection/time of killing | | | | | 24-h av[a] | Specific activity EGF injection at 1500 h (cpm × $10^{-3}$/mg DNA) | |
|---|---|---|---|---|---|---|---|---|
| | 0900/1300 h | 1500/1900 h | 1800/2200 h | 2100/0100 h | 0300/0700 h | | Experimentals | Controls |
| Animals killed 4 h after injection of EGF | | | | | | | | |
| Aorta | | 144 (<0.001) | | | | 23 | 8.3 ± 0.9 | 3.4 ± 0.3 |
| Cornea[b] | 369 | 52 | 287 | 43↓[c] | 65↓ | 31 | 6.1 | 4 |
| Cornea MI | 250 (<0.01) | | | 37↓ (<0.04) | 23 (<0.001) | 7 | 0.7 ± 0.1 | 0.2 ± 0.1 |
| Kidney | | 47 (<0.001) | | | | 8 | 6.9 ± 0.4 | 4.7 ± 0.4 |
| Liver | | 51 (<0.01) | | | | 1 | 12.1 ± 1.4 | 8.0 ± 0.6 |
| Lung | | 115 (<0.001) | | | | 19 | 11.4 ± 0.6 | 5.3 ± 0.4 |
| Parotid | 28↓ (<0.10) | 415 (<0.001) | | | | 59 | 17.0 ± 1.9 | 3.3 ± 0.2 |
| Testes | 90 (<0.02) | 53 (<0.10) | 40↓ (<0.10) | 45↓ (<0.10) | | 11 | 27.0 ± 5.5 | 17.6 ± 4.4 |
| Thymus | | 98 (<0.001) | | 14 (<0.18) | 22↓ (<0.01) | 15 | 16.6 ± 1.3 | 8.4 ± 0.4 |
| Bone Marrow | | 18↓ (<0.01) | | | 14↓ (<0.07) | 8↓ | 46.8 ± 3.9 | 57.3 ± 1.7 |
| Spleen | 25↓ (<0.09) | 21 (<0.06) | 38 (<0.08) | | 20 (<0.04) | 9 | 50.4 ± 5.1 | 41.5 ± 1.1 |
| | 0900/1700 h | 1500/2300 h | 1800/0200 h | 2100/0500 h | 0300/1100 h | | | |
| Animals killed 8 h after injection of EGF | | | | | | | | |
| Aorta | 35 (<0.02) | | 92 (<0.001) | | 23 (<0.08) | 29 | 4.0 ± 0.3 | 3.9 ± 0.2 |
| Cornea[b] | 33↓ | 64 | 157 | 66↓ | 47 | 24 | 7.4 | 4.5 |
| Cornea MI | | 300 (<0.001) | 59 (<0.004) | 141 (<0.001) | 48 (<0.002) | 79 | 3.2 ± 0.5 | 0.8 ± 0.2 |
| Kidney | 16 (<0.09) | 29 (<0.02) | | | | 4 | 7.2 ± 0.5 | 5.6 ± 0.5 |
| Liver | 20 (<0.04) | | 18↓ (<0.08) | | 32 (<0.06) | 10 | 10.5 ± 0.6 | 9.9 ± 1.2 |
| Lung | 35 (<0.01) | 30 (<0.01) | 56 (<0.01) | | 44 (<0.001) | 31 | 10.0 ± 0.6 | 7.7 ± 0.6 |
| Parotid | | 65 (<0.01) | 41 (<0.08) | 30↓ (<0.05) | | 2↓ | 5.3 ± 0.6 | 3.2 ± 0.5 |
| Testes | | 34↓ (<0.01) | | 123 (<0.04) | | 12 | 9.4 ± 1.3 | 14.2 ± 1.2 |
| Thymus | | | | 17↓ (<0.02) | | 4↓ | 9.6 ± 0.6 | 9.3 ± 0.6 |
| Bone Marrow | 14 (<0.10) | 18↓ (<0.02) | | 18↓ (<0.05) | | 8↓ | 46.3 ± 3.0 | 56.1 ± 2.5 |
| Spleen | | 34↓ (<0.001) | | 29↓ (<0.01) | 34↓ (<0.04) | 21↓ | 47.7 ± 3.2 | 72.0 ± 4.2 |
| | 0900/2100 h | 1500/0300 h | 1800/0600 h | 2100/0900 h | 0300/1500 h | | | |
| Animals killed 12 h after injection of EGF | | | | | | | | |
| Aorta | 59 (<0.01) | | | 29↓ (<0.05) | | 12 | 4.1 ± 0.3 | 3.5 ± 0.5 |
| Cornea[b] | 20↓ | 192 | 204 | 27 | 336 | 94 | 11.1 | 3.8 |
| Cornea MI | 400 (<0.01) | 131 (<0.002) | 40↓ (<0.001) | 48↓ (<0.001) | 85 (<0.001) | 10 | 3.0 ± 0.4 | 1.3 ± 0.1 |
| Kidney | | | | | 38↓ (<0.001) | 14↓ | 4.7 ± 0.6 | 5.5 ± 0.7 |
| Liver | 106 (<0.006) | 33 (<0.02) | 32 (<0.06) | 47 (<0.04) | | 36 | 12.1 ± 1.1 | 9.1 ± 0.6 |
| Lung | 48 (<0.02) | | | | | 10 | 6.5 ± 0.7 | 6.3 ± 0.9 |
| Parotid | 38 (<0.08) | | | | | 13↓ | 3.8 ± 0.6 | 6.1 ± 2.2 |
| Testes | | 42 (<0.05) | 26 (<0.09) | 75 (<0.07) | | 18 | 26.2 ± 2.5 | 18.4 ± 3.6 |
| Thymus | | 21↓ (<0.05) | 20 (<0.005) | | | 5↓ | 7.2 ± 0.7 | 9.1 ± 0.8 |
| Bone Marrow | 14↓ (<0.03) | 31↓ (<0.001) | 14↓ (<0.005) | | | 8↓ | 30.3 ± 1.3 | 44.1 ± 2.9 |
| Spleen | | 24↓ (<0.04) | | 22↓ (<0.03) | 17↓ (<0.05) | 15↓ | 38.3 ± 2.9 | 50.3 ± 5.1 |

Only those differences that are statistically significant or of borderline significance are shown. The specific activities measured for DNA incorporation are shown for one group of data to illustrate magnitude of values. Values in parentheses are *P* values.

[a] Comparison of 24-h means of control and experimental mice for each kill time, including all the nonsignificant sets of data. For example, the five mean control values and five mean experimental values for each tissue at 4, 8, or 12 h after injection were averaged and then compared on a percent basis. A "23" means that the experimental average along the 24-h scale is 23% greater than the control average, whereas "8↓" means that the experimental average is 8% less than the control average.

[b] Because of the small size of the cornea, it was necessary to pool all five corneas in order to obtain reliable values in DNA determination; therefore, no statistical analysis was possible.

[c] ↓, A decrease from control values; all other values represent increases.

the cornea (0900/1700 h; 2100/0500 h) and bone marrow (1500/2300 h; 2100/0500 h) and once in the liver (1800/0200 h, NS), parotid (2100/0500 h), testes (1500/2300 h), and thymus (2100/0500 h). Many of the inhibitory effects of EGF occurred at 8 h after the 2100 h injection (Table 1).

The greatest percent decreases in DNA synthesis in the affected tissues at 8 h after injection were 66% for the cornea (2100/0500 h), 34% for the spleen (1500/2300 h), 34% for the testes (1500/2300 h), 30% for the parotid (2100/0500 h), 18% for the liver (1800/0200 h, NS), 18% for the bone marrow (1500/2300 h; 2100/0500 h), and finally 17% for the thymus (2100/0500 h) (Table 1).

*Twelve hours after injection*

At 12 h after injection, EGF stimulated DNA synthesis four out of five times in the cornea (1500/0300 h; 1800/0600 h; 2100/0900 h; 0300/1500 h) and in the liver (0900/2100 h; 1500/0300 h; 1800/0600 h, NS; 2100/0900 h); three out of the five times in the testes (1500/0300 h; 1800/0600 h, NS; 2100/0900 h, NS); and, finally, once in the aorta (0900/2100 h), lung (0900/2100 h), parotid (0900/2100 h, NS), and thymus (1800/0600 h). EGF also stimulated the corneal epithelium mitotic index three out of the five times (0900/2100 h; 1500/0300 h; 0300/1500 h). At 12 h after injection, EGF did not stimulate DNA synthesis at any time in the kidney, spleen, or bone marrow (Table 1).

The greatest percent increases in DNA synthesis at 12 h after injection in the affected tissues were 336% for the cornea (0300/1500 h), 106% for the liver (0900/2100 h), 75% for the testes (2100/0900 h, NS), 59% for the aorta (0900/2100 h), 48% for the lung (0900/2100 h), 38% for the parotid (0900/2100 h, NS), and finally, 20% for the thymus (1800/0600 h). The greatest percent increase in the corneal epithelium mitotic index was 400% (0900/2100 h). Most of the greatest percent increases at 12 h after injection occurred after the 0900 h injection time, with killing at 2100 h (Table 1).

At 12 h after injection, EGF inhibited DNA synthesis three out of the five times in the bone marrow (0900/2100 h; 1500/0300 h; 1800/0600 h) and in the spleen (1500/0300 h; 2100/0900 h; 0300/1500 h). EGF also inhibited DNA synthesis at this time once in the aorta (2100/0900 h), kidney (0300/1500 h), and thymus (1500/0300 h). EGF inhibited the corneal epithelium mitotic index twice (1800/0600 h; 2100/0900 h) (Table 1).

The greatest percent decreases in DNA synthesis at

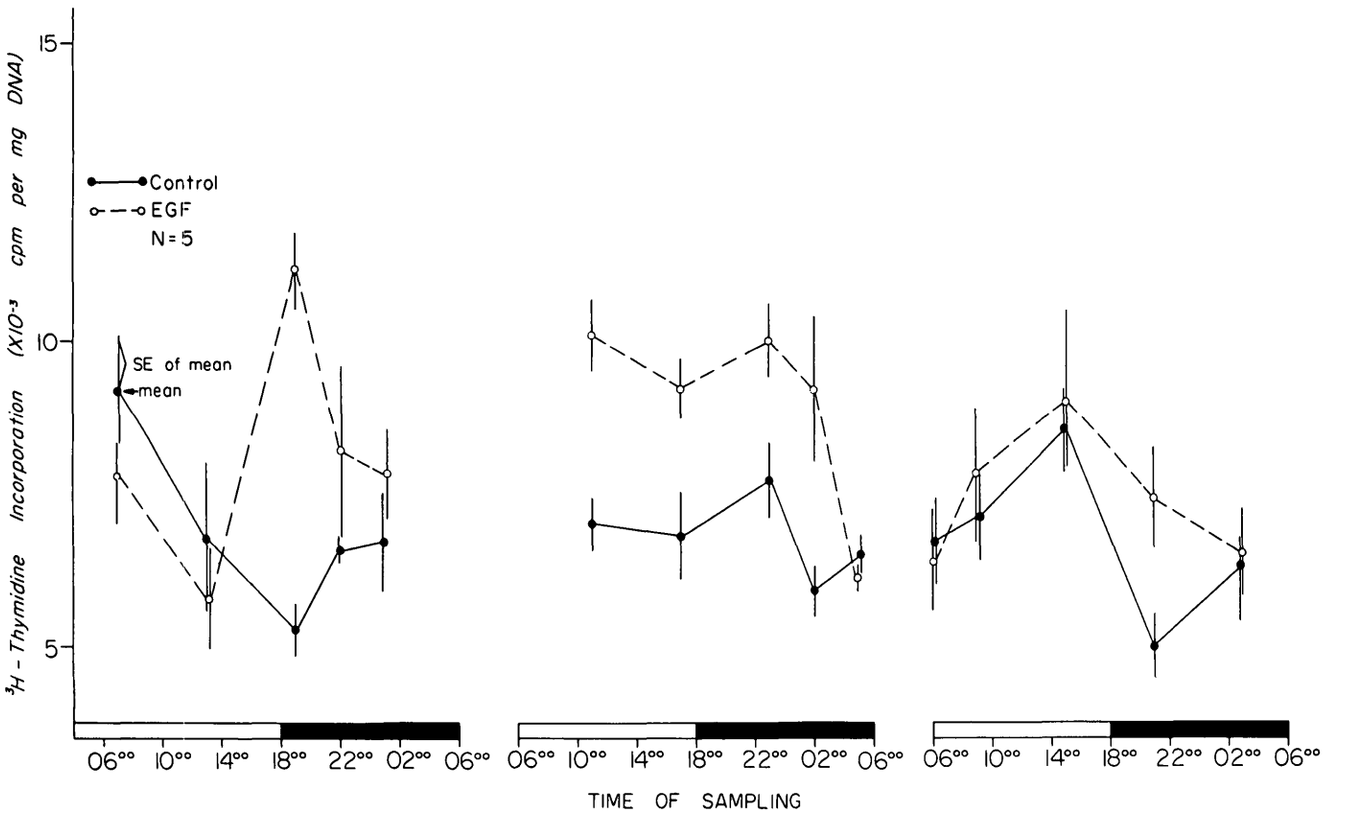


FIG. 1. EGF effect on [$^3$H]thymidine incorporation into the lung is plotted *vs.* time of sacrifice for the different injection times. Mice were killed at either 4, 8, or 12 h after injection for each injection time.

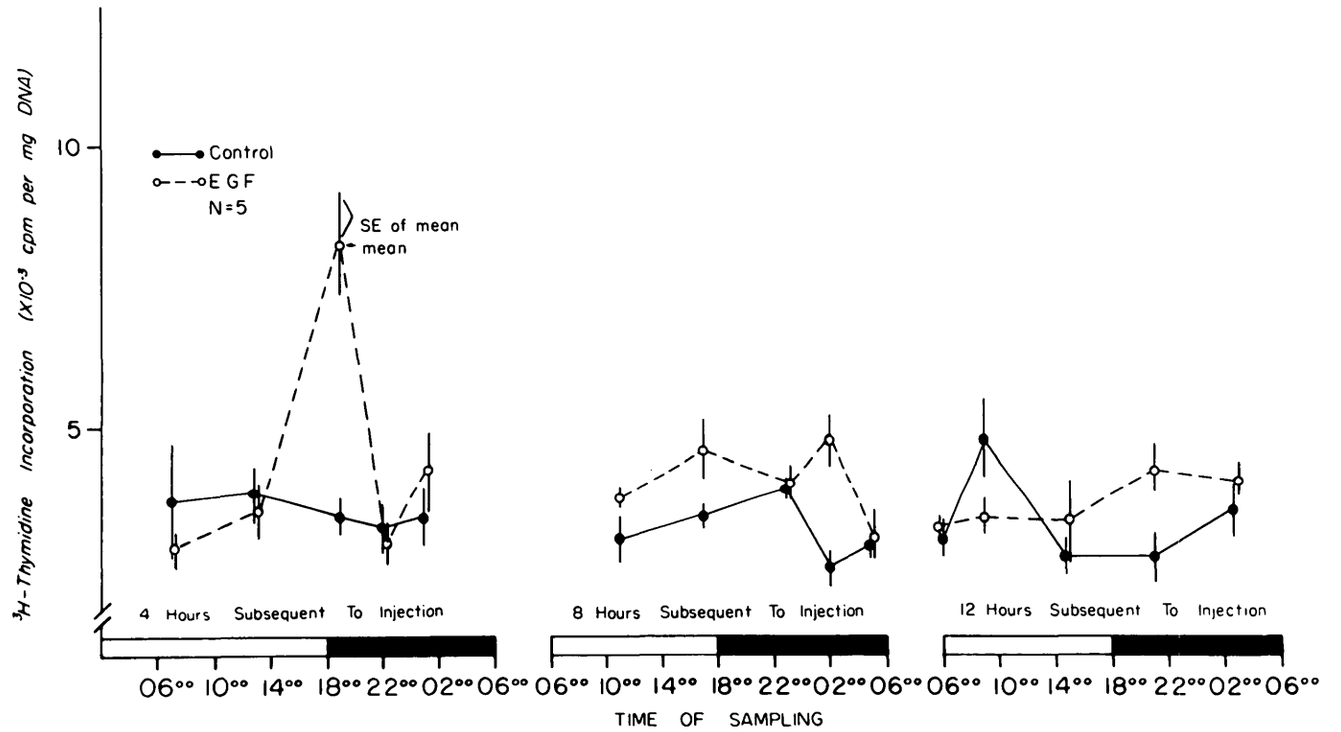


FIG. 2. EGF effect on [$^3$H]thymidine incorporation into the aorta is plotted *vs.* time of sacrifice for the different injection times. Mice were killed at either 4, 8, or 12 h after injection for each injection time.

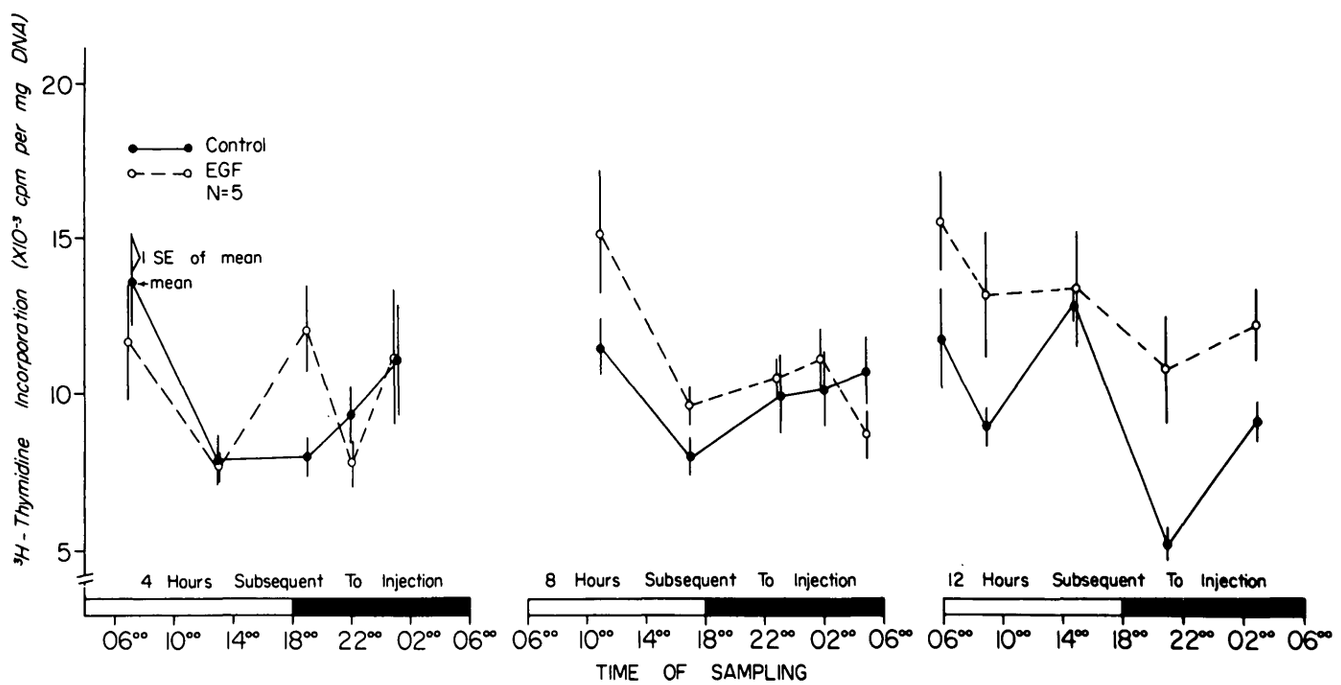


FIG. 3. EGF effect on [$^3$H]thymidine incorporation into the liver is plotted *vs.* time of sacrifice for the different injection times. Mice were killed at either 4, 8, or 12 h after injection for each injection time.

this time in the affected tissues were 38% for the kidney (0300/1500 h), 31% for the bone marrow (1500/0300 h), 29% for the aorta (2100/0900 h), 24% for the spleen (1500/0300 h), and 21% for the thymus (1500/0300 h). The greatest percent decrease for the corneal epithelium mitotic index was 48% (2100/0900 h). Note that most of the greatest percent decreases occurred at 12 h after either the 1500 h or 2100 h injections (Table 1).

## Discussion

In this paper we have presented data for the first time showing time-dependent effects of EGF on DNA synthesis in 10 different tissues as well as on the mitotic index of the corneal epithelium of the adult male mouse. The growth response of each tissue to EGF partly depended on the injection and kill times; however, EGF generally stimulated DNA synthesis in the cornea, lung, liver, aorta, testes, parotid, and kidney and inhibited it in the spleen, bone marrow, and thymus. The greatest stimulation of DNA synthesis by EGF for the different tissues at any given time-point was 415% for the parotid, 369% for the cornea, 144% for the aorta, 123% for the testes, 115% for the lung, 106% for the liver, 98% for the thymus, 47% for the kidney, 21% for the spleen, and 14% for the bone marrow. The greatest inhibition of DNA synthesis by EGF was 66% for the cornea, 45% for the testes, 38% for the kidney, 34% for the spleen, 31% for the bone marrow, 30% for the parotid, 29% for the aorta, 22% for the thymus, and 18% for the liver.

The responses of the various tissues to EGF differed with respect to the time after injection. For example, EGF stimulated DNA synthesis most consistently in lung and aorta at 8 h after injection (Figs. 1 and 2; Table 1) and in the liver and testes at 12 h after injection (Fig. 3; Table 1). In the spleen, EGF tended to stimulate DNA synthesis at 4 h after injection but inhibited it at 8 and 12 h after injection (Fig. 4; Table 1).

Others have studied the stimulatory effects of EGF on the growth of various tissues *in vivo*. Histological evidence has been presented that EGF promotes rabbit cornea wound healing, but a stimulatory effect of EGF on the growth of the normal rabbit cornea was not observed (12). EGF has also been shown histologically to promote the maturation of the lung of the fetal rabbit (4) and the growth of the kidney capsule, pericardium, liver, esophagus, tongue, and skin of the neonatal rat (13). EGF stimulated ornithine decarboxylase activity in the testes of the neonatal rat (14) and the stomach and small intestine of the neonatal mouse (15). Finally, the continuous ip infusion of EGF for 24 h slightly stimulated adult rat hepatic DNA synthesis, whereas the addition of either glucagon or insulin to the EGF infusion synergistically stimulated it, suggesting a role for such hormones in liver regeneration (5).

We hypothesize from all data available that EGF may play a role in the positive control of growth of numerous tissues; under the conditions of our investigation, this is particularly convincing for the cornea, liver, lung, testes, and aorta. Moreover, EGF may play an important role in the striking circadian rhythms of corneal DNA synthesis and corneal epithelium mitotic index (16) (Fig. 5), both of which have been shown in this study to be frequently, and at times dramatically, stimulated by exogenous EGF.

We have further demonstrated in this paper that EGF exerts an inhibitory effect on DNA synthesis in various tissues of the immune system, including the thymus, spleen, and bone marrow. This effect is as statistically significant as the above-mentioned stimulatory effect,

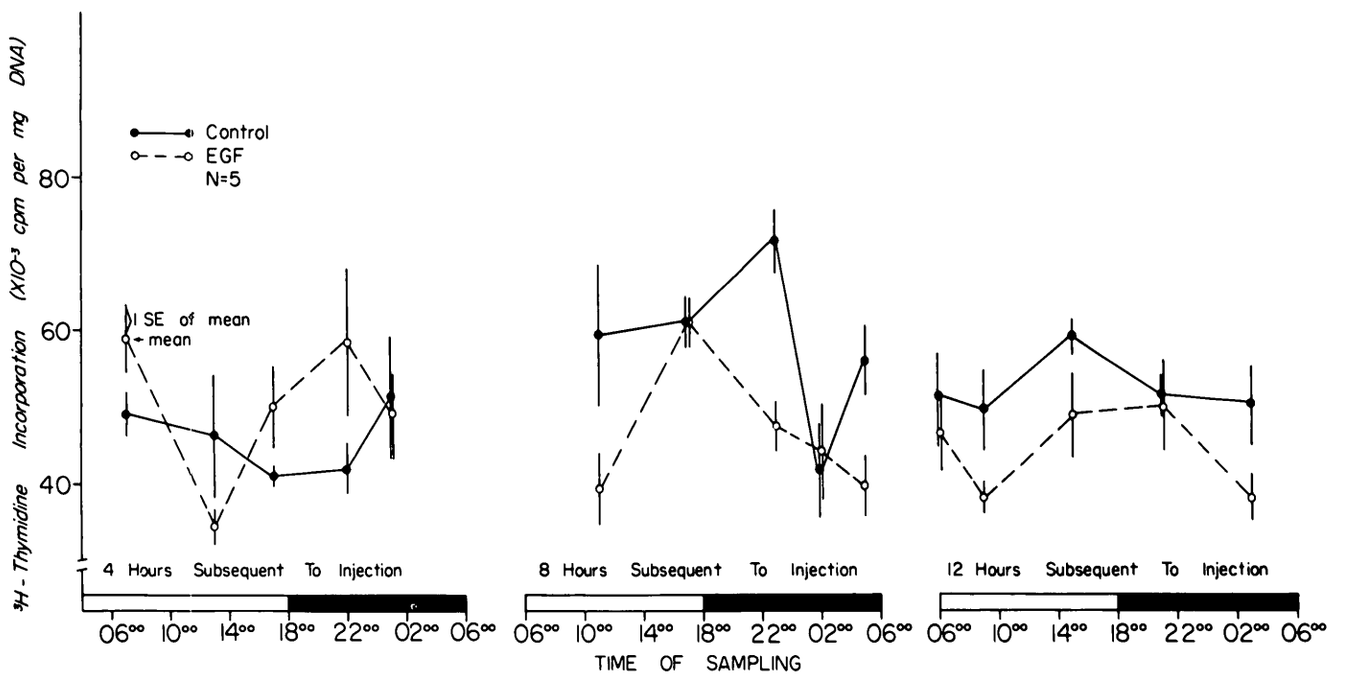


FIG. 4. EGF effect on [$^3$H]thymidine incorporation into the spleen is plotted *vs.* time of sacrifice for the different injection times. Mice were killed at either 4, 8, or 12 h after injection for each injection time.

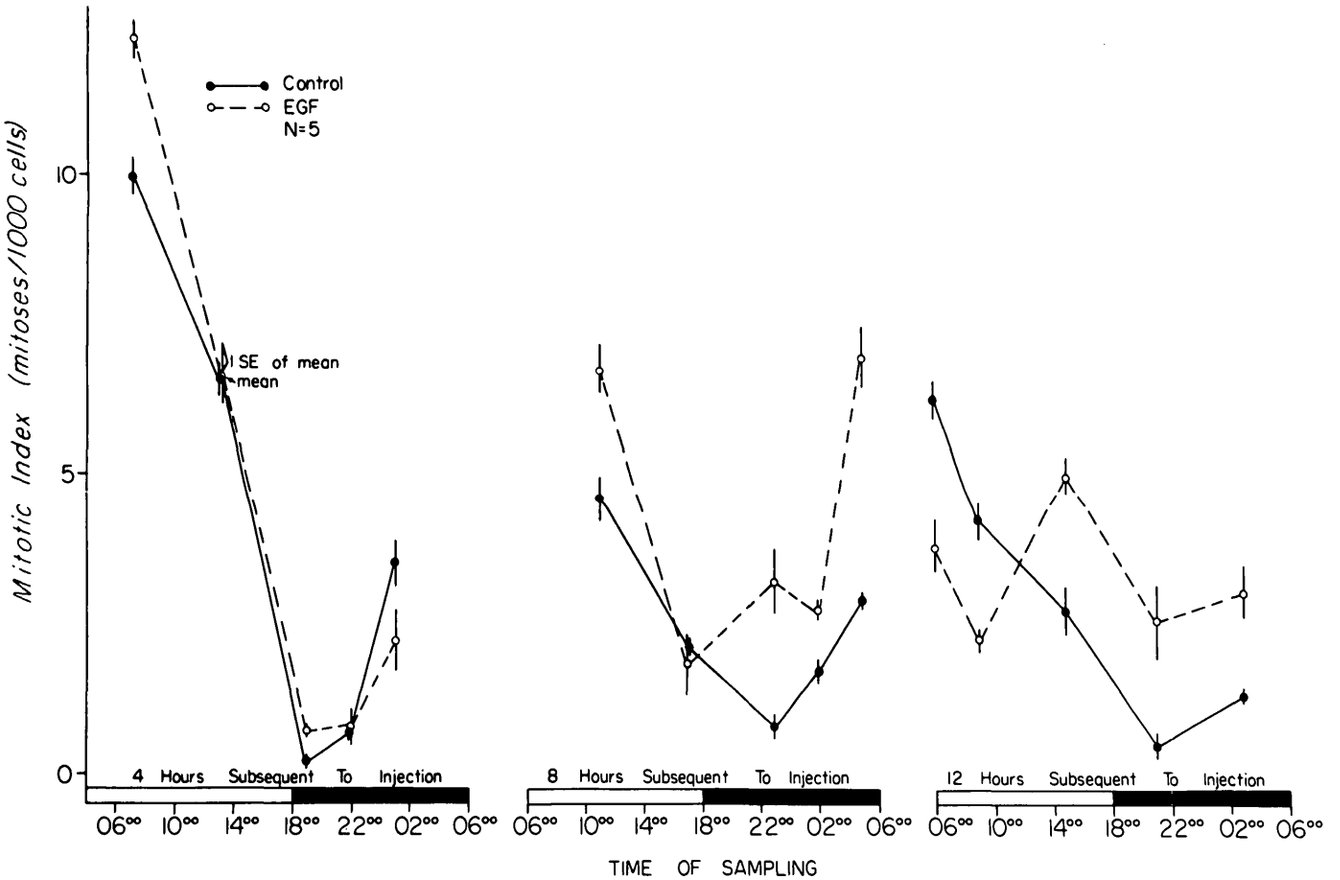


FIG. 5. EGF effect on [$^3$H]thymidine on the mitotic index of the corneal epithelium is plotted *vs.* time of sacrifice for the different injection times. Mice were killed at either 4, 8, or 12 h after injection for each injection time.

but it appears to be of smaller magnitude. This is noteworthy because there is considerable evidence that the submandibular gland contains an immunosuppressive factor: both submandibular gland extract and EGF depress the delayed-type hypersensitivity response to 2,4-dinitro-1-fluorobenzene in mice (17). Specific cell-surface receptors for EGF have been detected on thymic and splenic membranes (18). Submandibular gland extirpation results in thymic and splenic enlargement in male but not in female mice (19). Finally, one of the submandibular gland esteropeptidases, which has a molecular weight comparable to that of the EGF-binding protein (28,500), has been shown to induce thymic lymphocyte transformation both *in vitro* and *in vivo* (20). Further studies designed to better resolve the general inhibitory effect that EGF has on DNA synthesis in the thymus, spleen, and bone marrow *in vivo* therefore are warranted.

Finally, the time of injection affected the response of the various tissues to EGF. This point is most strikingly evidenced from the results obtained at 4 h after the injection at 1500 h or 1900 h, when the stimulatory effect of EGF was much greater than at 4 h after any of the other injection times. In this group of mice, DNA synthesis in mice treated with EGF increased relative to the controls by 415% in the parotid, aorta 144%, lung 115%, thymus 98%, testes 53%, cornea 52%, liver 51%, kidney 47%, and spleen 21%. In fact, the highest mean values in DNA synthesis in the parotid, aorta, lung, and thymus throughout the study were recorded for the group of mice injected with EGF at 1500 h and killed 4 h later at 1900 h. The only tissue that did not show an increase in DNA synthesis in this group of mice was the bone marrow in which, at this time, DNA synthesis was significantly decreased by 18%.

Several events occur during the period from 1500–1900 h that may have some bearing on the enhanced stimulatory effect of EGF on DNA synthesis in many tissues during this time. First of all, the increase in the rate of feeding and activity of the mouse occurs during this period, which bridges the transition from light to dark. Secondly, submandibular gland depletion of EGF begins toward the end of this period with the onset of darkness (21). Finally, the serum concentration of various nutrients, such as glucose (22), and hormones, such as insulin (22) and corticosterone (23) which exhibit prominent circadian variation, rise to the highest levels between

1500 h and 1900 h. The observation that insulin and EGF synergistically stimulate DNA synthesis of several cell types *in vitro* (24–26) and of hepatocytes *in vivo* (5) may therefore be relevant to the increased stimulatory effects of EGF on DNA synthesis at a time when serum insulin is rising. Other growth factors are no doubt also involved.

We point out that we have measured the incorporation of radioactive thymidine into DNA assuming that EGF does not cause any appreciable change in the nucleotide pool sizes and that thymidine incorporation is a reliable indicator of cell division. The terms "growth response" and "DNA synthesis" simply imply that there was a response in cell division.

In summary, the effect of EGF on DNA synthesis in 20 different tissues of the same adult male mice has been examined in this paper and in two previous ones (6, 7). Several conclusions have been reached. First of all, our results suggest that EGF plays an important role in the positive growth control of many tissues, particularly in the cornea and the digestive tract. Secondly, the different tissues exhibited considerable variation in their responses to EGF at 4, 8, and 12 h after injection. For example, the cornea, tongue, and esophagus consistently responded to EGF with increased DNA synthesis at 4, 8, and 12 h after injection; the glandular and nonglandular stomach, colon, and rectum at 8 and 12 h; the lung at 8 h; and the liver at 12 h. Thirdly, there are certain times when the stimulatory effect of EGF appears to be maximally potentiated. For example, the injection of EGF at 1500 h resulted in a striking generalized increase in DNA synthesis 4 h later at 1900 h, particularly in tissues such as the parotid, thymus, and small intestine, which at other times normally fail to respond positively to EGF. Fourthly, our results indicate that EGF can inhibit DNA synthesis at certain times in several tissues, including the small intestine, thymus, bone marrow, and spleen. Finally, the data point out the importance of considering the basic oscillatory behavior of DNA synthesis and mitosis when evaluating any growth-promoting or -inhibiting agent.

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