Table IV. Mitostatic activity of B 31 on mouse bone marrow cells in vivo

No. of mice	Compound injected i.p. (one single dose, mg/ml)	Lethality at the 3rd day	Mitoses % ° (mean values of 2 mice) after													
			2 hours					6 hours					18 hours			
			Total	P	M	A	T	Total	P	M	A	T	Total	P	M	A	T
6	—	0	11.5	3.0	4.5	2.5	1.5			n.d.					n.d.		
6	B 31 3000	5			n.d.					n.d.					n.d.		
6	B 31 1000	0	53	16.5	35.5	1	0	71	22	44.5	2.2	2.3	28.5	13	13.5	1	1
6	B 31 333	0	30.5	9.2	16.3	3	2	40	12.5	23.5	3	1	14	4	6	2	2
6	Vinblastine 10	6	92	13	76	2	1	105	17	88	0	0	60	10	50	0	0

n.d., not determined.

(Table I). Upon drug removal from the medium, the mitostatic effect of B 31 is easier to reverse than that of vinblastine[3] (Table II). Preliminary tests on structure-activity relationship have shown that compounds such as 4,6-dimethyl-2-amino-4-methoxyphenylpyrimidine (B 33), 2-amino-3,4,5-trimethoxyphenylpyrimidine (B 28) and 5-methyl-2-amino-3,4,5-trimethoxyphenylpyrimidine (B 32) have very little or no mitostatic effect on HEp2 cells, even at the maximum non-toxic concentrations for interkinetic cells (Table III). These data indicate that the mitostatic effect of B 31 calls for a trimethoxyphenyl ring and, in addition, for methyl groups, possibly in either or both 4,6 positions in the pyrimidine moiety.

Injected i.p. in mice, B 31 has a clear mitostatic effect on bone marrow cells. This in vivo effect, however, is far less pronounced than that observed in vitro, and is produced only by high doses of B 31 (Table IV).

Research is in progress to establish whether and which modifications in the B 31 structure may enhance the mitostatic effect. At present, B 31 can be considered as a promizing tool for cell synchronization.

[3] S. E. Malawista and K. G. Bensch, Science *156*, 521 (1967).

Epidermal Growth Factor Enhancement of Skin Tumor Induction in Mice

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Summary. Subcutaneous injection of epidermal growth factor 1. significantly shortened the latency period for the appearance of methylcholanthrene induced skin tumors and 2. increased the average number of papillomas elicited per mouse in both the Swiss Webster and C3HeB/FeJ strains.

A number of agents which stimulate hyperplasia in target tissues have been found to enhance the tumorigenicity of chemical carcinogens in these tissues. For example, in the rat mammary gland system the induction of tumors by combined administration of the chemical carcinogen methylcholanthrene (MCA), and prolactin, a protein hormone, has been well characterized[3]. Neither MCA nor prolactin administered individually could produce mammary tumors at the concentrations used.

A variety of physical and chemical agents[4–8], which have in common the ability to stimulate epidermal hyperplasia, have been reported to promote the induction of skin tumors by chemical carcinogens. Epidermal growth factor (EGF), isolated from the mouse submaxillary gland, is a polypetide growth factor which stimulates epidermal growth when injected into neonatal mice[9] and epithelial cellular proliferation when added to chick embryo skin fragments[10] and mouse mammary gland or mammary carcinoma[11,12] in organ culture. EGF also shares with phorbol myristate acetate, a potent promoter of skin tumor formation[13] derived from Croton oil, the ability to initiate cell division in non-dividing cultures of murine 3T3 cells[14,15].

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[2] Address reprint requests to Dr. H. Herschman.

[3] J. Furth, Fedn. Proc. *20*, 865 (1961).

[4] H. T. Deelman, Br. med. J. *1*, 872 (1927).

[5] H. Hennings and R. K. Boutwell, Cancer Res. *30*, 312 (1970).

[6] J. M. Twort and C. C. Twort, Am. J. Cancer *35*, 80 (1939).

[7] J. V. Frei and P. Stephens, Br. J. Cancer *22*, 83 (1968).

[8] W. F. Friedwald and P. Rous, J. exp. Med. *80*, 101 (1944).

[9] S. Cohen and G. A. Elliott, J. Invest. Dermat. *40*, 1 (1963).

[10] S. Cohen, Devel. Biol. *12*, 394 (1965).

[11] R. W. Turkington, Expl Cell Res. *57*, 79 (1969).

[12] R. W. Turkington, Cancer Res. *29*, 1457 (1969).

[13] B. L. Van Duuren and L. Orris, Cancer Res. *25*, 1871 (1965).

[14] A. Sivak, J. cell. Physiol. *80*, 167 (1972).

[15] S. P. Rose, R. M. Pruss and H. R. Herschman, J. cell. Physiol. *86*, 593 (1975).

A preliminary report by REYNOLDS, BOEHM and COHEN[16] indicated that EGF could enhance the induction of skin tumors by MCA in Swiss-Webster mice. Details of dosage, latency, numbers of papillomas, etc., are not provided in their report, however. Since we are interested in the possibility of obtaining cultured cells dependent on polypeptide growth factors, we have conducted a more detailed study of the ability of EGF to enhance MCA induced skin tumor formation in both the Swiss-Webster and the inbred C3HeB/FeJ strains of mice.

Materials and methods. EGF was prepared as previously described[15] except that prior to DEAE-cellulose chromatography the combined Sephadex G-100 fractions were heated at 85 °C for 10 min, cooled on ice, and centrifuged at 10,000 rpm for 10 min.

Females (18 to 20 days old) of the Swiss-Webster (Hilltop Labs., Scottsdale, Pa.) and C3HeB/FeJ (Jackson Labs., Bar Harbor, Maine) strains were shaved with surgical clippers. Control animals were injected s.c. on the lower back with 1 ml of 0.01 *M* potassium phosphate buffer (pH 7.0) containing 0.15 *M* NaCl (PBS), while experimental animals received 1 ml of PBS containing EGF (5 μg/g body weight). On the following day the mice were painted with a 0.6% solution of MCA (Sigma) in benzene using a No. 4 acrylic brush. Each mouse received a single brush stroke on either side of the vertebral column. Subsequently, all of the animals were painted once every 2 weeks with MCA. Injections with PBS (controls) or PBS containing EGF (experimentals) were given 6 days a week. The Swiss-Webster mice received 5 μg/g of EGF for the 1st week, 3 μg/g for the 2nd to 8th week and 2 μg/g for the subsequent weeks. In the Swiss-Webster study injections and paintings were terminated on the 17th week for the experimental group ($n = 12$) and on the 21st week for the control group ($n = 11$). The C3HeB/FeJ mice received 5 μg/g of EGF for the first 6 weeks and 3 μg/g for the subsequent weeks. In the C3HeB/FeJ study injections and paintings were terminated on the 20th week for control ($n = 10$) and experimental ($n = 10$) animals. Mice were examined weekly for the appearance of new papillomas. One of the C3HeB/FeJ experimental animals was sacrificed on the 18th week and 2 more on the 20th week for

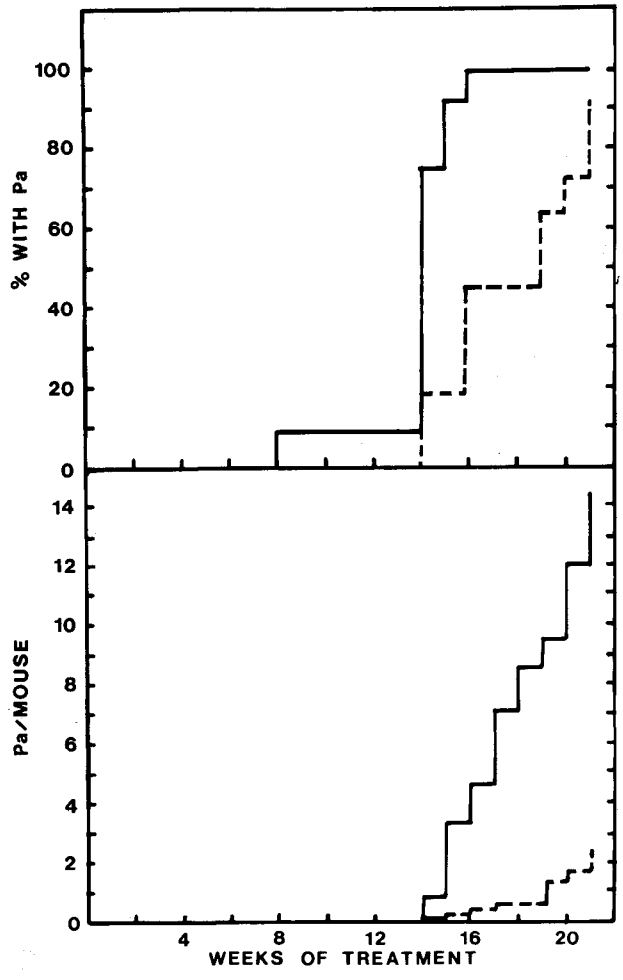

Fig. 1. The effect of EGF on the MCA induction of papillomas in Swiss-Webster mice. Control mice (dotted line) or experimental mice (solid line) received PBS injections or PBS containing EGF, respectively, 6 days a week. All animals were painted with MCA in benzene once every 2 weeks. The percentage of mice with papillomas (Pa) and the average number of papillomas per mouse (top and bottom graphs, respectively) are plotted as a function of weeks of treatment.

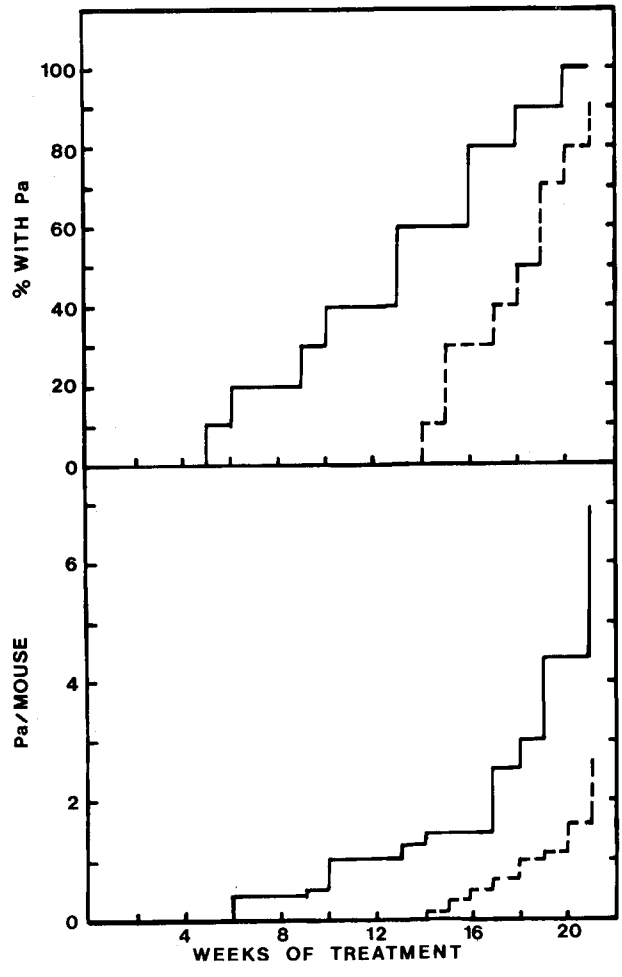

Fig. 2. The effect of EGF on the induction of papillomas by MCA in C3HeB/FeJ mice. See legend to Figure 1 for a description of the symbols and experimental details.

[16] V. H. REYNOLDS, F. H. BOEHM and S. COHEN, Surgical Forum *16*, 108 (1965).

preliminary studies of the growth properties of the papillomas in cell culture. Statistical analysis of the data was done using the two-sided WILCOXON Rank Sum Test[17].

Results and discussion. Figure 1 shows the effect of EGF treatment on the induction of skin tumors by MCA in Swiss-Webster mice. The time required for 50% of the mice to develop tumors was 19 weeks in the control group (PBS injections) but only 14 weeks in the animals injected with EGF. The time interval from initiation of the experiment to the appearance of the first tumor was significantly greater for the control group as compared with the experimental group ($p < 0.01$). In addition to shortening the latency period for the emergence of papillomas, EGF administration also caused a dramatic increase in the average number of papillomas per mouse throughout the course of this experiment (Figure 1, bottom). When the experiment was terminated at 21 weeks the EGF treated mice had an average of 6.0 times as many papillomas per mouse as the control animals. At 21 weeks the control Swiss-Webster mice all had between 0 and 5 papillomas per mouse. In the experimental group, one animal had 3 tumors while the rest of the group had between 10 and 20 tumors per mouse. The mean number of tumors were significantly greater for the experimental group as compared with the controls ($p < 0.01$).

Figure 2 shows the results of a similar experiment in C3HeB/FeJ mice. In this strain 50% of the experimental mice had developed tumors by 13 weeks (at which time none of the control mice had any tumors) while 50% tumor incidence in the control mice occurred at 18 weeks. For the C3HeB/FeJ experiment the time interval for papilloma appearance in the control group was also significantly greater than for the experimental group ($p < 0.02$). When this experiment was terminated the EGF treated mice carried an average of 2.6 times as many papillomas per mouse as the controls. For this strain at 21 weeks the control mice had from 0 to 6 papillomas per mouse, while the experimental animals had from 2 to 14 papillomas per mouse with the mean number of tumors being significantly greater for the experimental group ($p = 0.01$).

These studies clearly indicate that in both the Swiss-Webster and C3HeB/FeJ strains of mice EGF can enhance the carcinogenic activity of MCA, both in terms of the time of appearance of skin tumors and the average number of tumors which develop. Although our experiments do not exclude the possibility that EGF may itself be a carcinogen, attempts by others[18] to obtain papillomas in mice treated with EGF alone have been unsuccessful.

Our primary objective in these tumor induction studies has been to obtain EGF-sensitive tumors which could be adapted to growth in cell culture. We hope to obtain EGF dependent cell lines with appropriate selection procedures. The tumor induction studies reported here are necessary to define the time course for the appearance of tumors in EGF + MCA treated animals and to verify that there is, indeed, a significant difference between EGF + MCA treated mice and the MCA treated controls.

[17] F. WILCOXON and R. A. WILCOX, *Some Rapid Approximate Statistical Procedures* (Lederle Labs., Pearl River, New York 1964), p. 7.

[18] S. COHEN and R. SAVAGE, in *Recent Progress in Hormone Research* (Ed. R. O. GREEP; Academic Press, New York 1974), p. 572.

Calcium and Magnesium Deficiency-Induced Atrophy of Muscle and Calcium Accumulation in the Spinal Cord

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Summary. After severe dietary calcium-magnesium deficiency in rats, succinic dehydrogenase and acetylcholinesterase enzyme activity of gastrocnemius muscle showed a neurogenic atrophy. This alteration was associated with a high concentration of calcium in the spinal cord.

Recent clinical reports have suggested an interrelationship between a disturbance in calcium metabolism and the pathogenesis of neuromuscular disease[2,3]. Lacking reports on the neuropathology of the nervous system and muscles in the calcium and magnesium (Ca and Mg) deficient animal, we previously reported the changes in nervous system and related organs of experimental animals fed a Ca-Mg deficient diet[4].

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[2] Y. YASE, F. YOSHIMASU, Y. UEBAYASHI, S. IWATA and K. KIMURA, Proc. Japan Acad. *50*, 401 (1974).

[3] L. E. MALLETTE, B. M. PATTEN and W. K. ENGEL, Ann. intern. Med. *82*, 474 (1975).

[4] T. KUMAMOTO, S. NAKAGAWA, C. SUEMATSU, E. SHIMIZU, Y. YATA and T. HIROHATA, Acta Histochem. Cytochem. *8*, 294 (1975).

Effect of dietary calcium and magnesium restriction on concentration of calcium and magnesium in serum, spinal cord, gastrocnemius and kidney of rats

	Calcium		Magnesium	
	Control	Ca-Mg def.	Control	Ca-Mg def.
Serum (mg/100 ml)	8.68 ± 0.20	6.30 ± 0.29[a]	3.26 ± 0.19	1.54 ± 0.09[a]
Spinal cord (μg/g wet weight)	91.0 ± 10.7	286.6 ± 90.2	155.0 ± 5.2	149.1 ± 5.9
Gastrocnemius (μg/g wet weight)	46.9 ± 2.8	52.7 ± 6.9	277.3 ± 11.2	250.8 ± 10.8
Kidney (μg/g wet weight)	62.3 ± 4.7	1908 ± 245[a]	204.8 ± 8.8	184.2 ± 7.2

Each value is the mean ± SE of the mean of 14. [a] $p < 0.001$.