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EFFECT OF PUROMYCIN ON MITOTIC INDEX, AMINO ACID INCORPORATION AND DNA SYNTHESIS OF EPIDERMAL CELLS*

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ABSTRACT

Puromycin 200 mg/kg given systemically will block DNA synthesis, amino acid incorporation and mitosis in the epidermis of the baby rat. Topically applied solutions of 1% puromycin did not have any effect on uptake of H^3-thymidine or on mitosis of the epidermal cells. H^3-puromycin given intraperitoneally was demonstrated in the epidermal cells by radioautographs but no evidence of labeling of epidermal cells was found by topical application.

Puromycin is known to block protein synthesis after aminoacyl-s RNA is formed (1). The chemical structure of the amino acid-bearing end of aminoacyl-s RNA and puromycin are similar. During the course of protein synthesis, puromycin links to forming polypeptides giving a terminal peptidyl-puromycin. This reaction prevents further addition of peptide units and thus prematurely terminates synthesis of the protein (2).

Puromycin can prevent mitosis and DNA synthesis in vitro in epithelial cells of the lens (3) and in leukocytes stimulated with phytohemagglutinin (4).

Baserga *et al.* (5) reported immediate inhibition of protein synthesis and H^3-thymidine uptake when 2.5 mgm of puromycin was given intraperitoneally in mice. They detected no effect on thymidine kinase or DNA polymerase. Brotherton (6) reported a decrease in protein synthesis in pig skin cultures treated with puromycin.

Estenson and Baserga (7) injected 2.5 mgm puromycin per mouse every hour for 4 hours and injected H^3-thymidine, H^3-leucine and H^3-uridine 30 minutes before sacrifice. Uptake of H^3-thymidine and H^3-leucine was decreased 50–75% in the first 30 minutes after the first injection of puromycin. RNA incorporation increased for the first hour after puromycin, then decreased 25–50% in 2–4 hours.

Powell (8) showed that recovery of DNA synthesis under UVL exposure of mouse cells *in vitro* was prevented by puromycin. Twenty μg/ml of puromycin gave 80–90% inhibition of DNA synthesis but thymidine triphosphate levels remained unchanged. DNA polymerase activity was undisturbed by puromycin.

Mueller *et al.* (9) showed that *in vitro* puromycin will not interfere with DNA synthesis underway at time of addition of puromycin to culture. They suggest that DNA is in two states: (a) a fraction competent for replication and (b) a fraction requiring protein synthesis for initiation. They used 10 μg/ml puromycin.

Studzinski (10) showed that Hela cells with minimal effective concentration of puromycin (0.1 μg/ml) suffered some inhibition of mitoses at 24 hours. There was some accumulation of mitotic figures with 0.15 μg/ml for 24 hours. At 0.2–1.0 μg/ml there were no mitoses, thus indicating preprophase arrest.

The work that we wish to report concerns investigations of *in vivo* effects of puromycin on epidermal cells of baby rats and psoriatic skin. These studies are concerned with (1) the direct uptake of H^3-puromycin by epidermal cells when applied topically and when injected intraperitoneally in baby rats, (2) H^3-thymidine incorporation into DNA, amino acid incorporation and the mitotic index of baby rat skin and, (3) the mitotic index in psoriatic skin after topical application of puromycin.

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MATERIALS AND METHODS

Chemicals and their sources are listed below:

Puromycin—Nutritional Biochemical Co., Cleveland, Ohio.

H^3-puromycin—1800 mc/mM, Amersham/Searle, Des Plaines, Ill.

H^3-thymidine—11.6 Ci/mM, Amersham/Searle, Des Plaines, Ill.

H^3-leucine—15.0 Ci/mM, Schwarz BioResearch, Orangeburg, N.J.

H^3-histidine—5.1 Ci/mM, Schwarz BioResearch, Orangeburg, N.J.

Baby rats used were of the Sprague-Dawley strain. The rats were 3–5 days of age when started on the experiments.

Radioautographs were prepared using the method outlined by Epstein *et al.* (11).

I. *Effect of systemic puromycin on mitosis and DNA synthesis.* Baby rats were injected with puromycin 200 mg/kg or 10 mg/kg intraperitoneally at 8 a.m. and 9 a.m. One hour before sacrifice, 15 μc of H^3-thymidine was injected intraperitoneally. At given intervals the animals were sacrificed with ether. Samples of skin were taken from the dorsal neck area for fixation in Bouin's solution for determining mitoses or in buffered formalin for preparing autoradiographs. The number of mitoses and number of cells labeled with H^3-thymidine per 1000 interfollicular basal cells were determined for each animal. Control animals were injected with saline instead of puromycin but otherwise treated in the same manner. Twelve animals were taken for each study group.

II. *Effect of topical puromycin on mitosis and DNA synthesis.* 1% puromycin was applied to the back of baby rats in soaked cotton covering an area 4–5 mm in diameter and the cotton was covered with Blenderm® tape wrapped completely around the midsection of the animal. Water soaked cotton was used for controls. The wrapping was left in place for 20 hours. Twelve animals from two litters were used. Four of these animals were not included because the mother rat removed the Blenderm® tape.

H^3-thymidine (15 μc) was injected intraperitoneally 19 hours after initial application of the puromycin and the animal was sacrificed with ether one hour later. The mitotic index and labeling index were determined by counting mitoses and labeled cells per 1000 interfollicular basal cells.

1% puromycin was applied in soaked cotton to a 5–6 mm area of psoriatic skin of five psoriatic subjects. Both the puromycin and saline controls were covered with Blenderm® tape for 48 hours. The puromycin and saline soaked cotton were replaced at the end of 24 hours. Four mm biopsies were taken from each treated area, fixed in Bouin's solution and the mitotic index determined by counting the number of mitoses per 1000 interfollicular basal cells.

III. *Incorporation of intraperitoneal and topically administered H^3-puromycin.* 108 μc H^3-puromycin in 0.15 ml was applied to a 3 cm^2 area of the back of baby rats and allowed to dry. The area was then covered by Blenderm® tape and left in place for 7 and 24 hours. The animals were sacrificed with ether and 1 cm^2 areas of treated and untreated skin were removed and prepared for autoradiography.

108 μc H^3-puromycin in 0.15 ml was injected intraperitoneally into baby rats and the animals were sacrificed by ether inhalation three hours later. Skin from the back of the neck was fixed and prepared for autoradiographs.

IV. *Effect of puromycin on amino acid incorporation.* 100 mg/kg puromycin was injected subcutaneously on the ventral surface of young rats hourly for 3 hours or half-hourly for 5 hours before subcutaneous administration of H^3-histidine or H^3-leucine. Animals were killed with ether an hour later. Most of the dorsal skin of each animal was removed without including the subcutis. Skin specimens were immediately placed in carefully flattened aluminum foil envelopes, and the separation of epidermis from dermis was accomplished by placing the aluminum foil envelopes with dermis down on a slide warmer at 56° C for 30 seconds, under a paper pad and staining jar on which light manual pressure was maintained. Complete epidermal separation was then easily accomplished using clean forceps (histological confirmation of clean separation was obtained).

Epidermal specimens (100–150 mg) were then obtained from each rat, and approximately 10 mg portions (exact weight recorded) were obtained for scintillation counting and were treated by one of the following methods:

1. *Method A*

Washing consisted of two 15 minute immersions in 5% trichloracetic acid at room temperature, subsequent dipping in distilled water and drying on clean filter paper.

2. *Method B*

Duplicate epidermal specimens were taken from five baby rats treated with H^3-histidine and five treated with H^3-leucine. These specimens were subjected to the following sequence before solubilization of the epidermis to decrease the possible contamination with non-protein radioactivity:

(1) Homogenized epidermis in saline.
(2) Added equal volume of 10% TCA.
(3) Centrifuged and washed precipitate 3 × with 5% TCA.
(4) Suspended precipitate in 5% TCA and heated to 90 C for 15 minutes.
(5) Extracted precipitate with 50% ethanol, 100% ethanol, 50% ethanol/50% ether, and with ether.

Epidermal solubilization was then effected by using a technique suggested by representatives of the Beckman Co., Fullerton, California. Epidermal specimens (10 mg) were digested in 0.5 ml of 2 N NaOH in new scintillation counting bottles in an incubator at 80° C for 30 minutes. Bottles were removed to the bench, cooled to room temperature, and 1.5 ml of Beckman Bio-Solve® BBS_2 solubilizer

was added to each bottle from a Cornwall pipette. Gentle shaking produced a clear solution to which one drop of 0.5% stannous chloride was added, before adding 10 ml of Fluoralloy® Beckman scintillation cocktail. Specimens were then counted at ambient temperature in a Beckman LS 150 scintillation counter. The counting efficiency for tritium in solutions prepared in this manner was 30 per cent.

All statistical evaluations were based on the simple "t" test.

RESULTS

I. *Effect of systemic puromycin on mitosis and DNA synthesis.* Table I shows that the mitotic index is definitely depressed one hour after injection of puromycin 200 mg/kg ($p = <0.05$). There is almost complete suppression of mitoses in two hours ($p = <0.01$) and evidence of partial recovery after 24 hours. The 10 mg/kg dosage apparently was without influence on the mitotic index.

Table II gives evidence of definite suppression of DNA synthesis, as measured by H^3-thymidine incorporation, in 1.5 hours ($p = <0.05$) and a decrease of close to 90% by 3 hours ($p = <0.01$) with the 200 mg/kg dosage. There is a slight suggestion of recovery of DNA synthesis by 24 hours with about 80% suppression of H^3-thymidine uptake. The 20 mg/kg dose level was apparently without effect on H^3-thymidine incorporation.

II. *Effect of topical puromycin on mitosis and DNA synthesis.* 1% puromycin, topically, under occlusion, did not suppress either the mitotic index or H^3-thymidine uptake. Table III shows that topical 1% puromycin on psoriatic skin gives no significant evidence of suppression of mitosis.

III. *Incorporation of intraperitoneal and topically administered H^3-puromycin.* Radioautographs of baby rat skin 7 and 24 hours after intraperitoneal H^3-puromycin revealed diffuse labeling through the corium and epidermis. Some basal cells showed an increased density of labeling over the nucleus.

However, topical H^3-puromycin did not result in any detectable labeling of the corium or epidermis.

IV. *Effect of puromycin on amino acid incorporation.* Puromycin 100 mg/kg each hour for 3 hours or 5 hours resulted in a sharp drop in H^3 amino acid uptake by the epidermis ($p = 0.01$). The inhibition varied from 52–75% of the control values for leucine and histidine respectively (see Table IV).

TABLE I

*Mitoses per 1000 interfollicular basal cells**

Time post-injection	# of animals	Puromycin (200 mg/kg)	Puromycin (10 mg/kg)	Saline control
½ hour	12	4.1 (2–7)	4.3 (3–7)	5.0 (3–7)
1 hour	12	2.1 (1–5)	5.8 (4–8)	6.1 (5–8)
2 hour	12	0.3 (0–1)	7.1 (5–8)	6.0 (4–8)
3 hour	12	0.7 (0–1)	5.0 (4–7)	6.3 (5–9)
24 hour	12	3.1 (1–6)	7.0 (5–9)	6.1 (4–8)

* Expressed as average of all subjects with the extremes in parentheses.

TABLE II

*H^3 thymidine labeled basal cells/1000 interfollicular basal cells**

Time post-injection	# of animals	Puromycin (200 mg/kg)	Puromycin (20 mg/kg)	Saline control
1½ hours	8	45.2 (31–72)	71.2 (53–87)	64.5 (50–72)
3 hours	8	7.4 (2–12)	68.4 (54–78)	66.0 (48–75)
5 hours	8	7.8 (3–12)	62.2 (48–75)	58.1 (51–69)
7 hours	8	14.3 (6–23)	66.8 (54–80)	71.4 (56–89)

* Expressed as average of all subjects with the extremes in parentheses.

TABLE III

1% puromycin topically to psoriatic skin. Mitoses per 1000 interfollicular basal cells

Subject	Puromycin 1%	Saline
1	15	16
2	18	13
3	12	16
4	14	11
5	21	19
	—	—
	Avg. 16	Avg. 15

DISCUSSION

Intraperitoneal administration of 200 mg/kg of puromycin induced rapid and severe sup-

TABLE IV

The effect of puromycin hydrochloride on the incorporation of tritium labeled amino acids

Amino acid	Exp.	Puromycin dose and administration schedule	Mean DPM/mg of epidermal tissue	
			Treated group	Control group
H^3-histidine	1	1 mg/10 G rat each hour × 3	Method A 1337 Method B 1143	Method A 4047 Method B 3328
	2	1 mg/10 G rat each half-hour × 5	Method A 1640	Method A 4235
H^3-leucine	1	1 mg/10 G rat each hour × 3	Method A 775 Method B 682	Method A 3022 Method B 2601
	2	1 mg/10 G rat each half-hour × 5	Method A 1547	Method A 3211

Method A—treated group, 10 animals/experiment.
Method A—control (water-injected) group, 5 animals/experiment.
Method B—5 animals in treated and control groups.

pression of mitosis and DNA synthesis in epidermal cells of the baby rat. However, topical administration of 1% puromycin gave no indication of suppression of either mitosis or DNA synthesis. The fact that topical H^3-puromycin did not label epidermal cells but intraperitoneal H^3-puromycin did label them, could be explained by the fact that puromycin did not reach the basal cells when applied topically but did when given intraperitoneally. This may be a problem of penetration of the horny layer by puromycin or possibly metabolism of puromycin before it reaches the basal cells. Even in psoriatic skin, where presumably the barrier to penetration is damaged, topical puromycin did not depress the mitotic index.

A recent review by Baserga discusses the biochemistry of the cell cycle (12). It is not known precisely how puromycin interferes with DNA synthesis and mitosis but it is probably due to its inhibition of protein synthesis. Littlefield (13) has found that puromycin prevents the reappearance of thymidine kinase activity during the S-phase growth of mouse fibroblasts in culture. Kishimoto and Lieberman (14) demonstrated a puromycin sensitive step in the G_2 phase of mammalian cells in culture and Tobey *et al.* (15) reported on puromycin blocking a stage later in G_2 in Chinese hamster cells. In our study the suppression of the mitotic index 30 minutes after puromycin suggests a block in the G_2 phase. A previous report by Robinson and Stoughton (16) shows evidence for a very early suppression of mitosis by methotrexate. Estensen and Baserga (10) reported that crypt cells of the mouse intestine are inhibited in DNA synthesis by puromycin. They found DNA synthesis (S-phase) to be more sensitive to puromycin than G_2 or mitosis. They concluded that DNA synthesis seemed to be completely dependent on protein synthesis.

Baserga reported no change in thymidine kinase or DNA polymerase levels in mouse cells treated with 2.5 mgm per hour per animal (5). Although Estensen *et al.* (7) reported necrosis of actively dividing cells (small intestine, germinal centers of spleen and lymphoid tissue) treated with puromycin, we did not observe necrosis of rat epidermal cells with puromycin. These same authors (7) also reported a sharp decrease in amino acid uptake in the presence of puromycin.

The effect of puromycin on epidermal cell function is dramatic and rapid when the drug is given systemically. It is curious that there is no effect by topical application. This model system should be useful in the investigation of other antimetabolites for topical and systemic effects on epidermal cell proliferation.

Although Method B, which more critically excludes non-protein radioactivity than Method A, decreased the incorporated counts, the

puromycin effect of lowering amino acid incorporation was still quite evident.

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