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The *in Vivo* Effects of a Polyamine Analogue on Tissue Stem Cell Proliferation*

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Abstract— *We have studied by autoradiography the effects of* 1,3-*diaminopropane (DAP) upon cellular proliferation in a number of tissues* in vivo *in the rat. DAP is a structural analogue of the naturally occurring polyamine putrescine, and is believed to block cellular polyamine synthesis by supressing the induction of ornithine decarboxylase (the rate-limiting enzyme for polyamine biosynthesis). The continuous infusion of DAP into rats that had been partially hepatectomised prevented the subsequent waves of spermidine and DNA synthesis from taking place in the regenerating liver. The inhibition of DNA synthesis is accounted for primarily by a block in the entry of hepatocytes into S phase and not by a reduction in the rate of DNA synthesis itself. In contrast to the regenerating liver, DAP exerted minimal effects upon the proliferation of the gut epithelium and bone marrow elements. The proliferation of stem cells of these latter tissues, which are normally in a state of rapid and continuous proliferation unlike the liver, is thus much more resistant to perturbations in polyamine biosynthesis and function. DAP is consequently unable to arrest and so protect normal rapidly proliferating tissues from damage caused by anti-cancer drugs (e.g. hydroxyurea) that kill only proliferating cells. DAP cannot therefore be employed to selectively protect normal cells but not tumour cells from cytotoxic damage according to a principle we have previously established in tissue culture.*

INTRODUCTION

GROWTH stimulation of a variety of cell types both *in vivo* and in tissue culture is accompanied by increased activity of enzymes for polyamine biosynthesis and subsequent increases in the intracellular content of polyamines (reviewed in [1]). Polyamines appear to play a role in actually regulating the progression of cells through the cell cycle. Tissue culture studies demonstrate that normal fibroblasts from a number of species require polyamines for progression through G_1, i.e. the pre-DNA replicative phase of the cell cycle [2–4]. In contrast, bovine lymphocytes and hamster ovary cells exhibit a polyamine requirement for traverse of S phase, i.e. the DNA replicative phase itself, but not for progression through the preceding G_1 phase [5, 6]. Previous studies have demonstrated that inhibition of polyamine synthesis *in vivo* results in the subsequent inhibition of DNA synthesis in salivary gland [7] and liver [8] that accompanies growth stimulation of these tissues. However, an inhibition of DNA synthesis can be attributed to either a reduction in the proportion of cells in S phase (due to a block in the progression of cells through G_1) or a reduction in the rate of progression through S phase itself. Our studies define which of these two fundamentally different and operationally distinct mechanisms operates *in vivo*.

Our previous studies demonstrated that normal fibroblasts in tissue culture could be arrested in G_1 by treatment with an inhibitor of polyamine synthesis, while, in contrast, the tumorigenic counterpart of these cells continued to proliferate under the same conditions. Subsequent treatment of such cultures with an anti-cancer drug which is cytotoxic only to proliferating cells (e.g. hydroxy-

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urea) therefore resulted in the selective killing of the tumour cell population [9]. The use of this class of anti-cancer drugs is often accompanied by dose-limiting toxicity to normal rapidly proliferating tissues (e.g. bone marrow and gut epithelium) in the patient. Consequently we wished to determine whether this principle that we had established in tissue culture could be employed *in vivo* to selectively protect normal tissues but not tumours from anti-cancer drug toxicity.

MATERIALS AND METHODS

Partial hepatectomy and infusion procedures

Male Fisher rats weighing 240 g were partially hepatectomised under light ether anaesthesia and subsequently infused via a catheter, prepared from polyethylene tubing (Clay Adams PE50), inserted into the femoral vein. The catheter was connected to a syringe pump (Model 355, Sage Instruments) and the rats maintained in restraining cages for the duration of the experiment. Infusion solutions were pumped at a rate of 15 ml per rat per day starting 15–20 min after partial hepatectomy. The basic infusion mixture was a solution of glucose, electrolytes and amino acids [10]. Rats were either infused with this basic mixture alone or with the basic mixture supplemented with DAP (83 μmol DAP per rat per hr) for various times after partial hepatectomy performed at 0 hr. For reversal experiments rats were infused for 24 hr after partial hepatectomy with the DAP-supplemented mixture and then switched to the basic mixture lacking DAP infused for various times.

Measurement of polyamine content of tissue samples

The liver contents of putrescine, spermidine and spermine were determined by an amino acid analyser technique [11].

Autoradiography of tissues

Rats were injected i.p. with [^{3}H]-thymidine (125 μCi per rat; 40 Ci/mmol) and killed by heavy ether anaesthesia 1 hr later. Tissues were fixed in Carnoy's Fluid and autoradiograms prepared from histological sections (6 μm thickness) cut from wax-embedded tissue. Sections were coated with Kodak NTB3 Nuclear Track Emulsion, exposed for 1 month, developed and stained with haematoxylin and eosin.

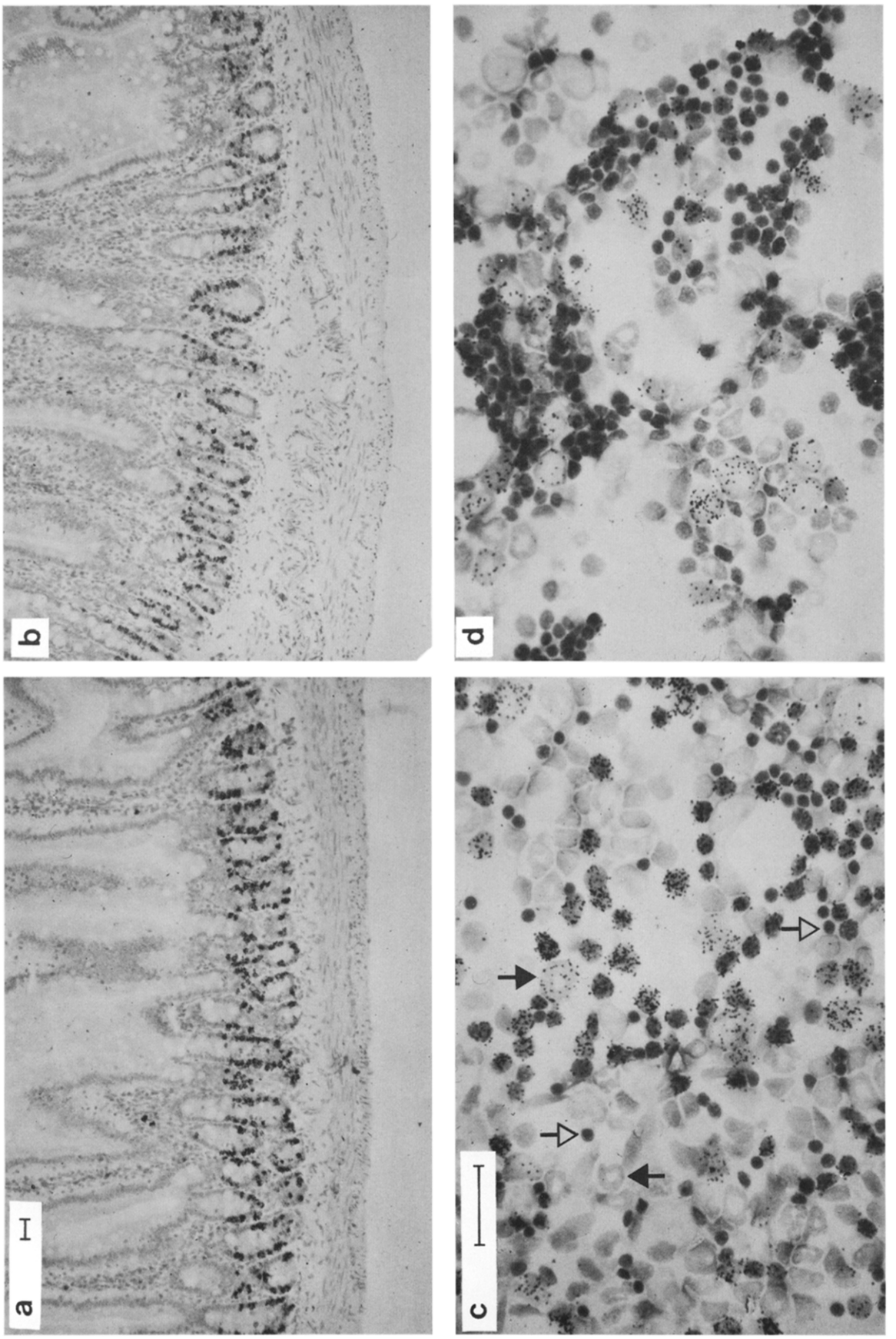
RESULTS

Effects of DAP on the regenerating rat liver

The synchrony of cellular proliferation in the regenerating rat liver makes this an ideal system for *in vivo* cell cycle studies. A sub-total (approx. 66%) hepatectomy is followed by the initiation of DNA synthesis in the liver remnant after a preparative lag period has been completed [12]. This lag phase is associated with increased RNA synthesis and the early induction of ornithine decarboxylase (ODC), the rate-limiting enzyme for polyamine biosynthesis, which results in increases in liver polyamine content [12, 13]. The changes in liver polyamine and DNA synthesis during regeneration are illustrated in Fig. 1a. Putrescine levels increase rapidly and subsequently decline as the putrescine is channelled into spermidine, while spermine levels remain relatively unchanged. The initiation of DNA synthesis occurs after a lag period of some 16 hr, as demonstrated by the synchronous entry of hepatocytes into S phase determined by autoradiography. These changes are in agreement with previous studies on polyamine metabolism [13] and kinetics of cellular proliferation [14–16] in the regenerating rat liver.

The administration of DAP by continuous infusion for 24 hr commencing immediately after partial hepatectomy effectively prevents the elevation of tissue spermidine normally observed in regenerating liver at this time (compare Fig. 1b, 24 hr and Fig. 1a, 24 hr). Because of the high dosage of DAP required to observe these effects and the similarity in physical properties of the inhibitor with the natural diamine putrescine, we were unable to satisfactorily quantitate these diamines individually. Although other methodologies enable the separation of putrescine and DAP [8], the polyamine whose content correlates with proliferative activity in this situation is spermidine [17]. In partially hepatectomised animals infused with DAP for 24 hr, the labelling index (0.7 ± 0.2%) is greatly reduced compared to that observed in control animals (12.5 ± 3.1%) but nevertheless significantly elevated over the labelling index in livers of unoperated animals (0.15 ± 0.05%). Since the intensity of [^{3}H]-thymidine labelling of nuclei is similar in control and DAP-treated animals, we conclude that the inhibition of DNA synthesis resulting from DAP treatment in regenerating liver [8] is accounted for primarily by a block in the entry of hepatocytes into S phase and not by a reduction in the rate of DNA synthesis or rate of traverse of S phase itself.

When partially hepatectomised rats infused for 24 hr with DAP are switched to the control infusion mixture lacking DAP, the labelling index rises rapidly after an initial lag period of 5–9 hr (Fig. 1b), further illustrating the arrest of hepatocytes in the G_1 phase of the cell cycle as a result of DAP treatment. The labelling index, however, does not reach the levels observed in control animals (Fig. 1a), indicating that DAP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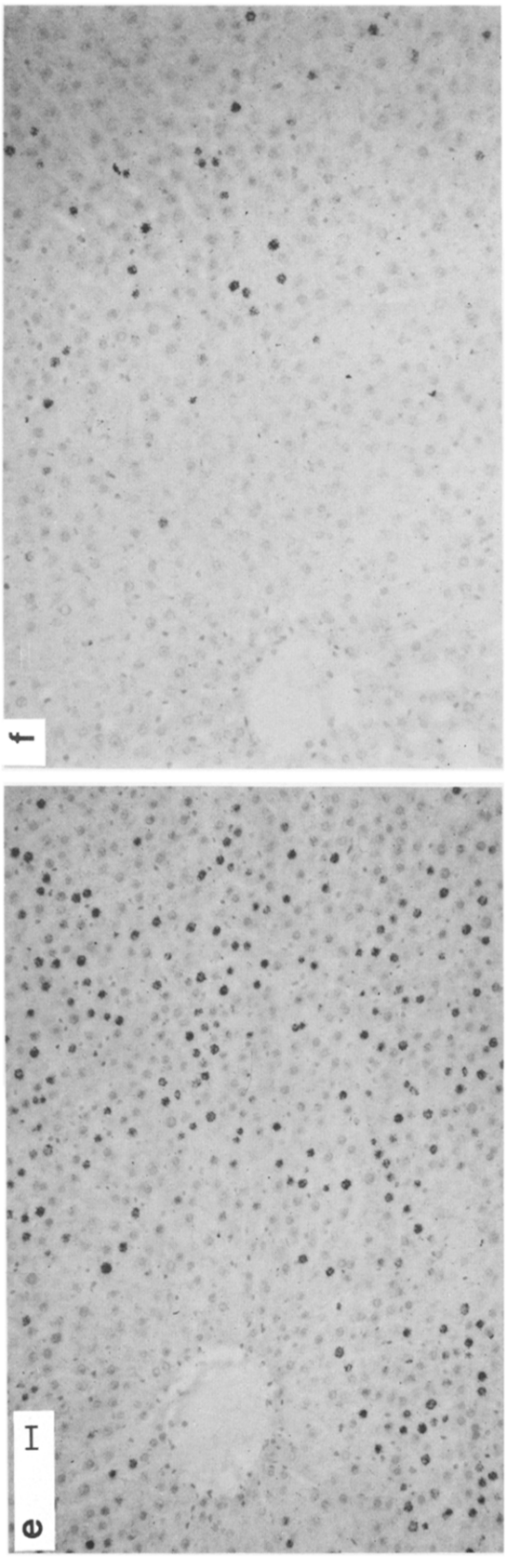

Fig. 2. Autoradiographic study of the effects of DAP treatment upon the proliferation of various rat tissues. Autoradiography was performed on tissues from rats that had been partially hepatectomised 24 hr earlier and then pulsed for 1 hr with [^{3}H]-thymidine. Autoradiographs are depicted for small intestine (a), bone marrow (c) and liver (e) from control animals, together with the corresponding tissues (b, d and f) from rats that were infused with DAP for 24 hr immediately following partial hepatectomy. Cells typical of the granulocytic or white cell lineage (solid arrows) and erythrocytic or red cell lineage (open arrows) are indicated in (c). The bar represents 25 μm.

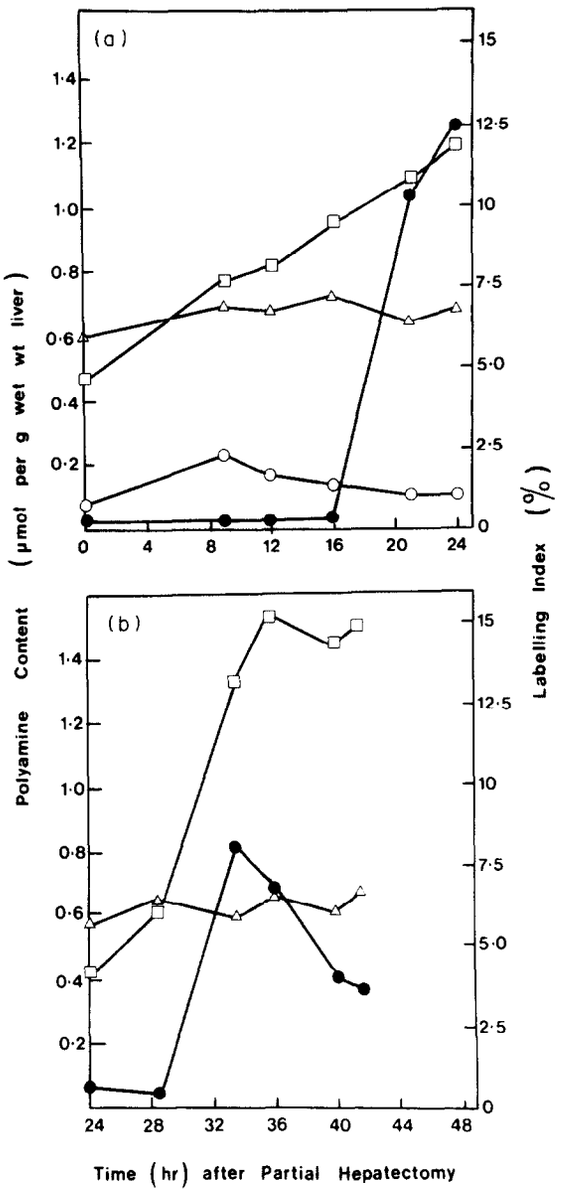

Fig. 1. The effects of DAP upon the labelling index (●) and polyamine levels, putrescine (○), spermidine (□) and spermine (△), of regenerating rat liver. Following partial hepatectomy performed at 0 hr, *rats were infused with either (a) the basic infusion mixture alone lacking DAP for various times or (b) the infusion mixture supplemented with DAP for* 24 hr *and then switched to the basic mixture without DAP for various times. Labelling index determinations were restricted to the large polygonal cells constituting hepatocyte population* [16]. *Each value depicted is the mean derived from data obtained from a minimum of* 3 *rats; S.E.M. within* 25% *of mean for polyamine determinations and S.E.M. within* 35% *of mean for labelling index measurements.*

treatment has some irreversible character, at least over the time period studied here. The shortened lag phase (Fig. 1b) before cells enter S phase following release from DAP treatment compared to control animals (Fig. 1a) suggests that DAP may arrest hepatocytes at some point rather late in the G_1 phase of the cell cycle. The entry of cells into S phase correlates with a rapid elevation in liver spermidine levels following release from DAP treatment (Fig. 1b).

The effects of DAP upon the gut epithelium and bone marrow

The gut epithelium lining the small intestine consists of a continuously and rapidly dividing cell population localised in a proliferative zone in the crypt at the base of each villus that replaces mature and maturing cells found in the upper portions of the villi (reviewed in [18]). The continuous infusion of DAP for 24 hr elicited only marginal effects upon the proportion of stem cells in the intestinal crypts that incorporated [^{3}H]-thymidine into DNA (labelling index) and the extent of incorporation of [^{3}H]-thymidine into DNA (intensity of labelling) (compare Figs 2a and 2b). The bone marrow represents another continuously and rapidly dividing tissue and generates the various blood cell types that appear in the systemic circulation (reviewed in [19]). Again, DAP exerted only minimal effects upon the incorporation of [^{3}H]-thymidine into DNA, irrespective of whether the cells were precursors in the white or red blood cell lineages (compare Figs 2c and 2d). The effects of DAP upon the proliferation of gut epithelium and bone marrow were similar in both partially hepatectomised and unoperated animals (unpublished observations). While there was a tendency towards a reduction in both the labelling index and intensity of labelling of cells in the gut epithelium and bone marrow as a result of DAP treatment, these effects were clearly minimal and in marked contrast to the effects of DAP upon hepatocyte proliferation in the regenerating liver (compare Figs 2e and 2f).

DISCUSSION

The present studies clearly demonstrate that inhibition of spermidine synthesis in the regenerating rat liver by DAP is associated with a marked inhibition of the entry of hepatocytes into S phase. Subsequent removal of DAP suggests that polyamines may be required at some point late in G_1 in order for hepatocytes to progress further through the cell cycle and enter S phase. While the inhibition of entry of hepatocytes into S phase correlates with an inhibition of spermidine synthesis, the effects of DAP probably cannot be attributed entirely to its role as an inhibitor of polyamine synthesis. DAP is classically considered to inhibit polyamine synthesis by mimicking the effects of high levels of natural polyamines in suppressing the induction of ODC, an effect attributed to the close structural similarity of DAP to the natural polyamines [20, 21]. Consequently, DAP may also interfere with some normal functions performed by the

polyamines present in the cell, irrespective of whether or not the polyamine content of the cell is changing, by virtue of its being a structural but not necessarily a functional analogue of the natural polyamines. Thus, in addition to its role as an inhibitor of polyamine synthesis, a further role for DAP should be considered, for example, as an inhibitor of protein synthesis [22, 23].

A most significant observation of this study is the minimal perturbation induced in the proliferation of the gut epithelium and bone marrow by DAP under conditions where liver proliferation was nevertheless almost completely inhibited by the polyamine analogue. It appears to be much more difficult to manipulate the rate of proliferation of continuously and rapidly dividing tissues as compared to quiescent tissues that must be stimulated in order to induce proliferation of the normal resting or non-dividing cell population. The studies of Luk and co-workers employing another inhibitor of polyamine synthesis, DL-α-difluoromethyl-ornithine (DFMO), appear to corroborate our observations. DFMO, a potent direct inhibitor of ODC, was found to suppress the polyamine accumulation and histological regeneration that accompany the recovery process following injury to the intestinal mucosa of adult rats [24]. In contrast, DFMO suppressed basal ODC activity in the intestinal mucosa of normal adult rats, but no histological changes resulted in this tissue as a consequence of the inhibition of polyamine synthesis [24]. Although the ineffectiveness of DAP in the gut epithelium may be attributable to the high diamine oxidase activity in this tissue [25], this enzyme is associated much more with the maturing rather than the proliferating cells of the intestinal epithelium [26]. Polyamine synthesis appears, in fact, to be associated primarily with maturing rather than proliferating cells in the intestine [26]. Thus our studies with DAP and those of Luk *et al.* [24] with DFMO would indicate that regenerative as opposed to basal proliferation rates are more closely regulated by tissue polyamine levels.

The gut epithelium and bone marrow do not therefore appear to behave in a manner analogous to hepatocytes *in vivo* or normal fibroblasts in culture following treatment with inhibitors of polyamine synthesis [3, 4]. Such agents can selectively arrest the proliferation of normal but not tumour cells in tissue culture studies and so selectively protect the normal cells from subsequent treatment with an anti-cancer drug specifically cytotoxic for proliferating cells [9]. However, since DAP does not arrest the proliferation of the normal rapidly dividing tissues that are most susceptible to such cytotoxic damage *in vivo*, DAP cannot be employed as an agent to protect these tissues according to the principle that we had established in tissue culture.

While our attempts to modulate the proliferation of continuously dividing tissues *in vivo* with DAP were discouraging, a more aggressive or prolonged interference with polyamine metabolism may prove to be effective. We considered DAP to be inappropriate for such studies because of its severe toxicity when employed over a 48-hr period (unpublished observations). Also, DAP would appear not to be the most effective agent available in view of the recent finding that DAP can, in fact, partly take over the role played by the polyamines whose synthesis it is inhibiting [27]. This drawback probably applies also to the action of other diamines since 1,5-diaminopentane and 1,6-diaminohexane, both more potent inhibitors of ODC induction than DAP [20, 28], had minimal effects upon the proliferation of bone marrow and gut epithelium when infused over a 24-hr period (unpublished observations). Although our study does not represent a definitive determination of whether polyamines can modulate the proliferation of the gut epithelium and bone marrow, it does contrast the ease with which liver proliferation can be modulated with the refractoriness of continuously dividing tissues.

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REFERENCES

1. JANNE J, POSO H, RAINA A. Polyamines in rapid growth and cancer. *Biochim Biophys Acta* 1978, **473**, 241–293.
2. BOYNTON AL, WHITFIELD JF, ISAACS RJ. A possible involvement of polyamines in the initiation of DNA synthesis by human WI-38 and mouse BALB/3T3 cells. *J Cell Physiol* 1976, **89**, 481–488.
3. RUPNIAK HT, PAUL D. Regulation of the cell cycle by polyamines in normal and transformed fibroblasts. In: CAMPBELL RA, ed. *Advances in Polyamine Research*. New York, Raven Press, 1978, Vol. 1, 117–126.
4. RUPNIAK HT, PAUL D. Inhibition of spermidine and spermine synthesis leads to growth arrest of rat embryo fibroblasts in G_1. *J Cell Physiol* 1978, **94**, 161–170.

5. FILLINGAME RH, JORSTAD CM, MORRIS DR. Increased cellular levels of spermidine or spermine are required for optimal DNA synthesis in lymphocytes activated by concanavalin A. *Proc Natl Acad Sci USA* 1975, **72**, 4042–4045.
6. SUNKARA PS, RAO PN, NISHIOKA K. Putrescine biosynthesis in mammalial cells: essential for DNA synthesis but not for mitosis. *Biochem Biophys Res Commun* 1977, **74**, 1125–1133.
7. INOUE H, KATO Y, TAKIGAWA M, ADACHI K, TAKEDA Y. Effect of DL-α-hydrazino-δ-aminovaleric acid, an inhibitor of ornithine decarboxylase, on polyamine metabolism in isoproterenol-stimulated mouse parotid glands. *J Biochem* 1975, **77**, 879–893.
8. POSO H, JANNE J. Inhibition of polyamine accumulation and deoxyribonucleic acid synthesis in regenerating rat liver. *Biochem J* 1976, **158**, 485–488.
9. RUPNIAK HT, PAUL D. Selective killing of transformed cells by exploitation of their defective cell cycle control by polyamines. *Cancer Res* 1980, **40**, 293–297.
10. BUCHER NLR, SWAFFIELD MN. Regulation of hepatic regeneration in rats by synergistic action of insulin and glucagon. *Proc Natl Acad Sci USA* 1975, **72**, 1157–1160.
11. SHULL KH, MCCONOMY J, VOGT M, CASTILLO A, FARBER E. On the mechanism of induction of hepatic adenosine triphosphate deficiency by ethionine. *J Biol Chem* 1966, **241**, 5060–5070.
12. BUCHER NLR. Regeneration of mammalian liver. *Int Rev Cytol* 1963, **15**, 245–300.
13. RAINA A, JANNE J, HANNONEN P, HOLTTA E. Synthesis and accumulation of polyamines in regenerating rat liver. *Ann NY Acad Sci* 1970, **171**, 679–708.
14. FABRIKANT JI. The kinetics of cellular proliferation in regenerating liver. *J Cell Biol* 1968, **36**, 551–565.
15. GRISHAM JW. A morphological study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver. *Cancer Res* 1962, **22**, 842–849.
16. YAGER JD, HOPKINS HA, CAMPBELL HA, POTTER VR. An autoradiographic analysis of DNA synthesis following partial hepatectomy in rats. In: LESCH R, REUTTER W, eds. *Liver Regeneration After Experimental Injury*. New York, Stratton Intercontinental Medical Book Corporation, 1975, 56–60.
17. KALLIO A, POSO H, JANNE J. Inhibition of prereplicative polyamine accumulation in regenerating rat liver. *Biochim Biophys Acta* 1977, **479**, 345–353.
18. LIPKIN M. Proliferation and differentiation of gastro-intestinal cells. *Physiol Rev* 1973, **53**, 891–915.
19. PATT HM, QUASTLER H. Radiation effects on cell renewal and related systems. *Physiol Rev* 1963, **43**, 357–396.
20. HELLER JS, CHEN KY, KYRIAKIDIS DA, FONG WF, CANELLAKIS ES. The modulation of the induction of ornithine decarboxylase by spermine, spermidine and diamines. *J Cell Physiol* 1978, **96**, 225–234.
21. POSO H, JANNE J. Inhibition of ornithine decarboxylase activity and spermidine accumulation in regenerating rat liver. *Biochem Biophys Res Commun* 1976, **69**, 885–892.
22. KAY JE, BENZIE CR. Direct effects of 1,3-diaminopropane on reticulocyte protein synthesis. *FEBS Lett* 1980, **121**, 309–312.
23. TUOMI K, RAINA A, MANTYJARVI R. 1,3-Diaminopropane rapidly inhibits protein synthesis and virus production in BKT-1 cells. *FEBS Lett* 1980, **111**, 329–332.
24. LUK GD, MARTON LJ, BAYLIN SB. Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. *Science* 1980, **210**, 195–198.
25. LUK GD, BAYLESS TM, BAYLIN SB. Diamine oxidase: a circulating marker for rat intestinal mucosal maturation and integrity. *J Clin Invest* 1980, **66**, 66–70.
26. BAYLIN SB, STEVENS SA, SHAKIR KMM. Association of diamine oxidase and ornithine decarboxylase with maturing cells in rapidly proliferating epithelium. *Biochim Biophys Acta* 1978, **541**, 415–419.
27. ALHONEN-HONGISTO L, POSO H, JANNE J. Inhibition of polyamine accumulation and cell proliferation by derivatives of diaminopropane in Ehrlich ascites cells grown in culture. *Biochim Biophys Acta* 1979, **564**, 473–487.
28. GUHA SK, JANNE J. Inhibition of ornithine decarboxylase *in vivo* in rat ovary. *Biochem Biophys Res Commun* 1977, **75**, 136–142.