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# Evolution of Red Blood Cell Polyamine Levels in Partially Hepatectomized Rat

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**Abstract**—*Erythrocyte levels of polyamines, especially of spermidine are greatly elevated during the course of liver regeneration. In contrast to putrescine and spermine levels, from the tenth hour to the fourth week after partial hepatectomy, correlation has been observed between the elevation of liver and erythrocyte spermidine concentrations. Substituting drinking water with* 2% α *difluoromethylornithine* (α-*DFMO*) *commencing* 48 *hr prior to partial hepatectomy and continuing until death, though ineffective in inhibition of liver* [$^3H$]*-thymidine incorporation, prevented the rise in hepatic putrescine concentrations* (*without modifying those of spermidine and spermine*) *and correlatively decreased red blood cell* (*RBC*) *spermidine levels. Thus, an excess of liver spermidine produced from an excess of newly synthetized putrescine could be released in blood and taken up by erythrocytes, especially as affinity of RBC for spermidine is at least* 30 *fold higher than that for putrescine.* In vivo *the spermidine half-life in RBC was estimated to be* 2.5–3.0 *hr, which could explain the elevation of erythrocyte and liver spermidine levels. The elevation of erythrocyte spermidine concentration is not correlated to that of the regenerating liver weight but dependent on the extent of partial hepatectomy. The elevation of erythrocyte spermidine concentrations, which appeared to be linked to the cellular proliferating activity, could contribute to determine intratumoral proliferation in cancerous patients.*

## INTRODUCTION

NUMEROUS studies have been steadily accumulating evidence that polyamines play a crucial role in cell proliferation [1–4]. Correlation between stimulation of cell growth and increases in the rate of polyamine biosynthesis has been previously studied during the course of liver regeneration [5]. In regenerating rat liver, ornithine decarboxylase (ODC) activity triples 1 hr after a two-third partial hepatectomy and is 10 times that of control levels by the fourth hour, resulting in concomitant increase in the concentration of liver putrescine [6, 7]. Then high putrescine levels activate adenosylmethionine decarboxylase [8], which generates an aminopropyl group needed to convert putrescine into spermidine, and spermidine into spermine [9].

In this animal experimental model, the cellular functions of these natural occuring polyamines seem closely associated with the cell proliferation process [10]. Indeed, as recently shown by Poso and Pegg [11], the use of αdifluoromethylornithine (α-DFMO), an irreversible potent inhibitor of ODC [12, 13], greatly inhibits the DNA synthesis in the regenerating liver remnant.

Moreover, as recently reported in clinical studies, increased concentrations of polyamines in biological fluids of the organism may provide a diagnostic means to evaluate tumor activity [14–16]. Circulating polyamines appear to be principally transported in the blood by erythrocytes [17], and high polyamine levels have been found in red blood cells (RBC) of patients harboring histologically confirmed malignant hepatic tumors [18], intracranial [19] or bronchopulmonary [20] cancers, and in mice bearing melanoma [21] or the Lewis lung (3LL) carcinoma [20].

In an attempt to define the relationship existing between cell proliferation and erythrocyte polyamine levels, we studied the correlations between RBC and liver polyamine concentrations in partially hepatectomized rat.

## MATERIALS AND METHODS

*Chemicals*

[1,4-$^{14}$C]-Putrescine dihydrochloride (sp. radioactivity 122 mCi/mmol), [$^{14}$C]-spermidine (*N*-(3-aminopropyl)-[1,4-$^{14}$C]-tetramethylene-1,4-diamine trihydrochloride; sp. radioactivity 122

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mCi/mmol), [$^{14}$C]-spermine (*N,N'*-diamine tetrahydrochloryde; sp. radioactivity 122 mCi/mmol) and [6-$^{3}$H]-thymidine (sp. radioactivity 23 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. α-DFMO was a generous gift from Merrell International Research Centre, Strasbourg, France. All other biochemical reagents were products of Sigma Chemical Company,, St Louis, MO, U.S.A.

*Rats*

Two hundred and thirty-five male rats of the Wistar-Commentry strain were locally obtained, maintained under a 12 hr lighting schedule (the dark period beginning at 8:00 pm) and used when weighing about 200 g.

Partial hepatectomy was performed in 210 animals under light ether anesthesia; they consisted of either 68% resection (two-third hepatectomy) as described by Higgins and Anderson [22], or approximately 33% resection (one-third partial hepatectomy) by excision of the left lateral lobe [23]. Twenty-five sham-operated animals were anesthetized and laparotomized. In some experiments, rats were given drinking water containing 2% α-DFMO commencing 48 hr prior to two-third partial hepatectomy and continuing until death.

In any case, rats were killed at 16:00 pm in groups of five by beheading and the blood was collected in a 0.129 M sodium citrate solution; the regenerating liver remnants were weighed and stored at −80° C until assayed for their polyamine contents or DNA specific activity determination.

*Polyamine extraction*

*Red blood cell (RBC) polyamines.* Blood samples were centrifuged at 2500 ***g*** during 10 min at +4° C. After removal of plasma and the buffy coat layer, the cell pellet was washed three times with 4 volumes of 0.14 M NaCl. One ml of RBC pellet was carefully removed and hemolysed with 2 ml of distilled water. An RBC pellet aliquot was taken in order to evaluate the number of erythrocytes with a Thoma counter. Protein was removed from the hemolysate by the addition of 2 ml of ice cold 10% $HClO_4$. After shaking and centrifugation at 3000 ***g*** for 10 min, the supernatant was frozen at −40° C.

*Liver samples.* For biochemical assay, the frozen samples were homogenized in 10 volumes of ice cold 10% $HClO_4$ in motor-driven glass homogenizers and maintained for 2 hr at 0–2° C. The homogenates were then centrifuged at 5000 ***g*** for 10 min at +4° C. The supernatants were frozen at −40° C.

*Polyamine determination*

To each 1 ml of perchloric extract was added 500 μl of a saturated aqueous solution of $Na_2CO_3$ then 1 ml of a dansyl chloride solution (5 mg/ml acetone). After shaking, the tubes were left overnight opened under a dark fume-hood to allow selective evaporation of the acetone [24]. The dansylated derivatives were extracted twice with 1 ml each of benzene and evaporated to dryness at 50° C on a rotary evaporator. The residues were redissolved in acetonitrile and subjected to HPLC according to SAEKI's techniques [25]. The mobile phase for elution was a linear gradient between 20% acetonitrile in water (V/V) and acetonitrile. Analyses were performed on a 25 × 0.26 cm column packed with 10 μm C-18 reversed phase packing (Spherisorb S50DS). Measurements of fluorescence intensity was performed on an LCD fluorimeter.

In vitro *polyamines uptake by RBC incubated with DFMO*

Washed erythrocytes were dispensed into plastic tubes in aliquots of 500 μl containing $4.10^9$ cells. To each of the aliquots of RBC was added 2 nmol of [$^{14}$C]-putrescine, spermidine or spermine in a volume of 50 μl, and 500 μl of homologous serum. After gentle shaking at 37° C for 1 hr, RBC were centrifuged and the pellet washed three times with 0.14 M NaCl, which was sufficient to eliminate extracellular radioactivity. The RBC were then lysed by addition of 2 volumes of distilled water and protein precipitated from the hemolysate with 2 volumes of ice cold 10% $HClO_4$. After centrifugation, the radioactivity present in 50 μl aliquots of the supernatants was evaluated on a Packard tricarb spectrometer with a toluene ethanol scintillation mixture. Thin layer chromatography of the dansylated $HC10_4$ supernatant was also carried out as previously described [26] to assess whether the labelled polyamine added had been converted to other polyamines. As previously observed [27], no evidence for interconversion was obtained.

In an attempt to study the influence of α-DFMO dissolved in 50 μl of 0.14 M NaCl at the end of the incubation period, the RBC were processed as described above.

Results are expressed as pmol of $^{14}$C-polyamine incorporated by $4.10^9$RBC during a 60 min period.

*DNA specific activity*

DNA synthesis was measured as previously described [11] by the incorporation of radioactivity into DNA during a 60 min period after injection of 5 μCi [6-$^{3}$H]-thymidine. DNA content was determined by the method of Burton [28]. Radio-

activity in aliquots from the acid-insoluble fraction was counted in PCS scintillation fluid (Amersham Corp., Arlington Heights) for 5 min at 20% efficiency; quench correction was by internal standard. Results were expressed as DPM incorporated by μg DNA.

*Statistical method*

The non parametric U test of Mann and Whitney was used for statistical evaluation of the data.

## RESULTS

Erythrocyte levels of polyamines (Fig. 1), especially of spermidine (Fig. 1B), are significantly enhanced during the course of liver regeneration. It can be observed that within the second and fifth hour after a two-third hepatectomy, RBC putrescine and spermine levels are double than normal values (Fig. 1A and C) whereas spermidine values are six times those of controls (Chart 1B). In contrast to putrescine and spermine levels, from the tenth hour to the fourth week after operation, a statistically significant correlation ($r = 0.96$; $P < 10^{-6}$) has been observed between the elevations of liver and erythrocyte spermidine concentrations (Fig. 1B). It must be noticed that the early and markedly increased RBC spermidine level (2.5 hr after partial hepatectomy) does not occur in the regenerating liver remnant, and that [$^3$H]-thymidine incorporation begins from the 18th hour after operation (Fig. 1D). Despite an enhancement of RBC putrescine and spermidine level 2.5 hr after laparotomy (Table 1), this sham operation has only statistically modified the liver concentrations of putrescine during the first 10 hr after surgical intervention.

Taking these results into consideration two main questions remain: first, is this increase of RBC spermidine level related to newly synthetized putrescine in the liver of partially hepatectomized rat? Second, is RBC spermidine level elevation dependent on the importance of the regenerating process? In response to the first question, two-third partially hepatectomized rats were given drinking water containing 2% α-DFMO (as indicated in Materials and Methods section) starting 48 hr prior to the operation. In an attempt to determine the relationship existing between RBC spermidine concentration and the level of hyperplastic process, we performed one-third partial hepatectomies.

As previously shown by Poso and Pegg [11], though α-DFMO is ineffective via oral administration in inhibition of liver [$^3$H]-thymidine incorporation (Fig. 1D), this drug is able to prevent the rise of hepatic putrescine concentrations (Fig. 1A) without modifying those of spermidine (Fig. 1B) and spermine (Fig. 1C). Yet, during the first 10 hr of liver regeneration, RBC spermidine levels are significantly reduced concomitantly to those of putrescine concentrations in the liver of α-DFMO-treated rats (Fig. 1A and B). We observed a similar effect 48 hr and 72 hr after surgery, the decrease of hepatic putrescine concentrations in α-DFMO-treated animals being once more associated with lowered RBC spermidine levels.

Polyamines being—at least *in vitro* [27]—not metabolized in normal erythrocytes, α-DFMO could modify the take-up of putrescine, spermidine and spermine by RBC.

As shown in Table 2, in spite of the fact that polyamines are slowly taken up by erythrocytes when incubated with α-DFMO, it cannot explain by itself the reduced spermidine take-up by RBC in partially hepatectomized rats treated with this drug. Indeed, as indicated by our experiments, we should have observed lower putrescine and spermine erythrocyte concentrations. α-DFMO being rapidly cleared from the blood of the rat [29], it is tempting to speculate that RBC spermidine levels could be related to a lower putrescine synthesis in regenerating rat liver treated by this inhibitor of ODC.

Do liver and erythrocyte spermidine concentrations depend on the extent of partial hepatectomy, thus of the hyperplastic process? As is the case for a two-third partial hepatectomy, or even for a laparotomy [30], one-third partial hepatectomy is responsible for a rise in the ODC activity [31]. As shown in Table 1, in one-third partially hepatectomized rats we observed a rise of RBC spermidine levels proportional both to the extent of the hepatectomy and to liver concentrations of spermidine. Therefore, 2.5 hr after operation RBC spermidine levels are of 35 nmol/$8.10^9$ RBC instead of 60 nmol/$8.10^9$ RBC after a two-third hepatectomy (Fig. 1B). During the following hours, it is still possible to observe as we did, a correlated elevation of spermidine levels in the liver and erythrocytes.

From the tenth hour on, after a two-third partial hepatectomy, RBC spermidine levels are constantly correlated to hepatic spermidine ones, and not to the wet weight of regeneration liver remnant (Fig. 1D). Interpretation of these results indicates a possible relationship between the evolution of the hyperplastic process and that of spermidine levels in erythrocytes.

## DISCUSSION

As previously reported in mice bearing the Lewis lung (3LL) carcinoma [20], in the course of experimental liver regeneration, from the tenth hour on, a correlation between hepatic and RBC spermidine levels elevation can be established. This polyamine seems to play an important role in the course of cell proliferation, and it has been recently shown

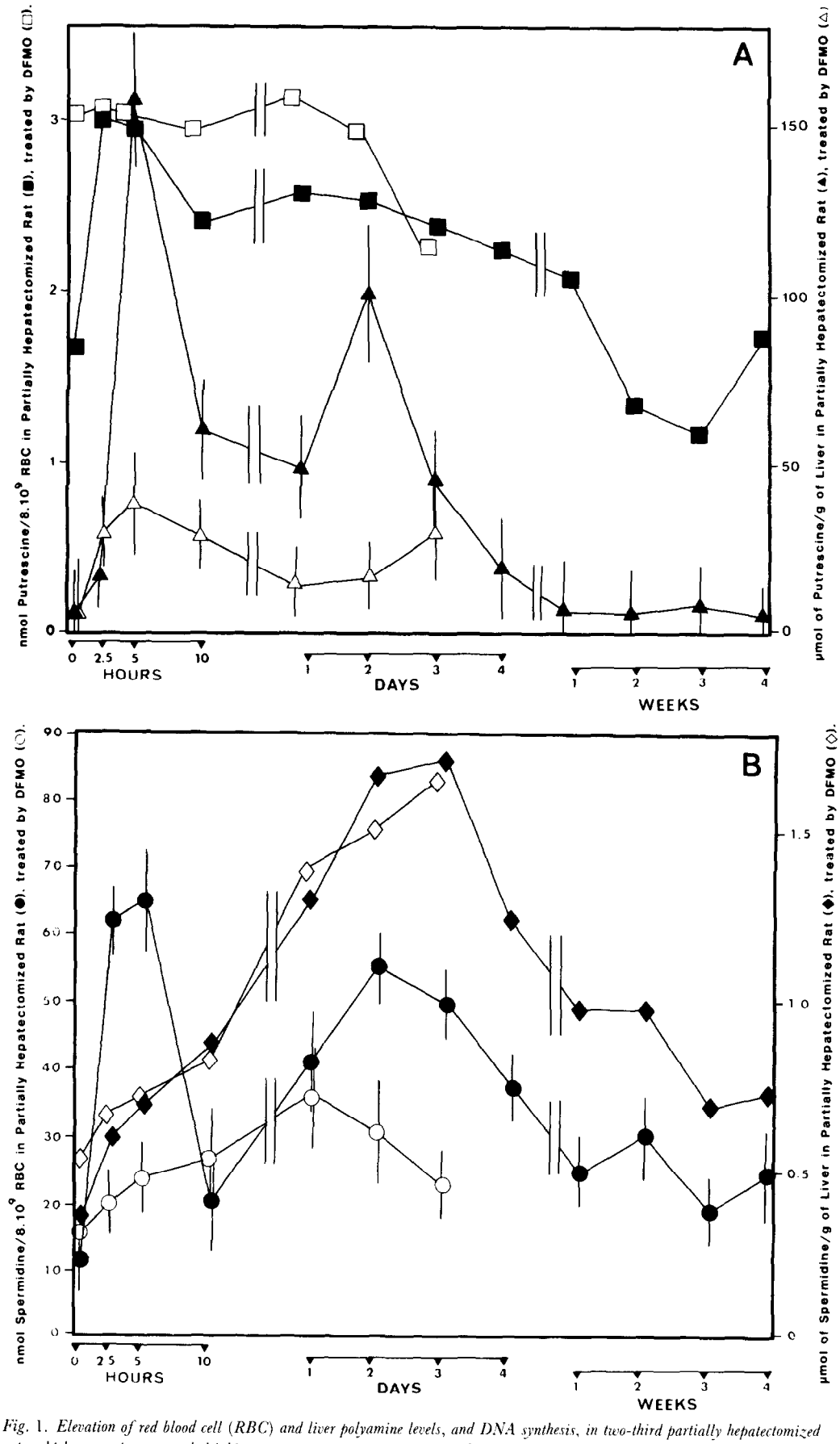
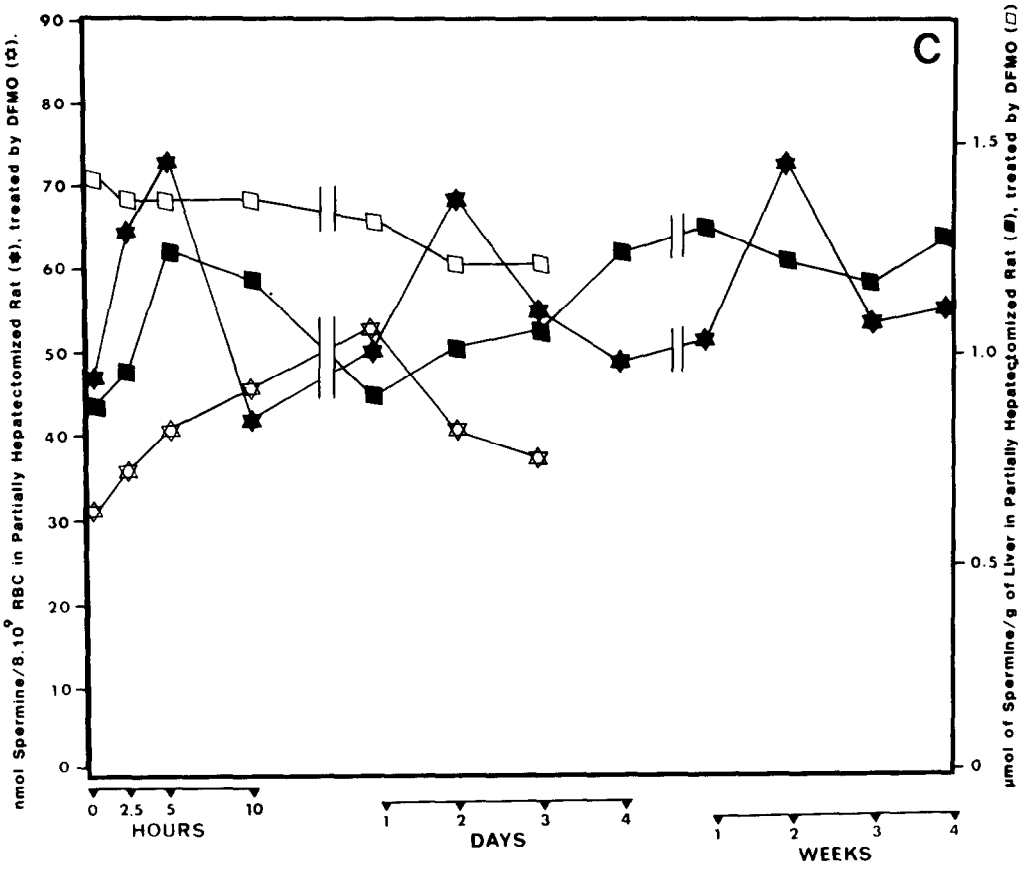



*Fig. 1. Elevation of red blood cell (RBC) and liver polyamine levels, and DNA synthesis, in two-third partially hepatectomized rats which were given normal drinking water or water containing 2% DFMO starting 48 hr prior to surgery and continuing until death. Results are shown as means ± S.D. for five animals for putrescine (Panel A), spermidine (Panel B) and spermine (Panel C) concentrations, and for DNA synthesis (Panel D). No statistically significant differences have been noticed during the evolution of RBC putrescine and spermidine levels observed in two-third partially hepatectomized rats treated or not by DFMO.*


C
nmol Spermine/8.10⁹ RBC in Partially Hepatectomized Rat (✱), treated by DFMO (✡).
µmol of Spermine/g of Liver in Partially Hepatectomized Rat (■), treated by DFMO (□)
HOURS
DAYS
WEEKS

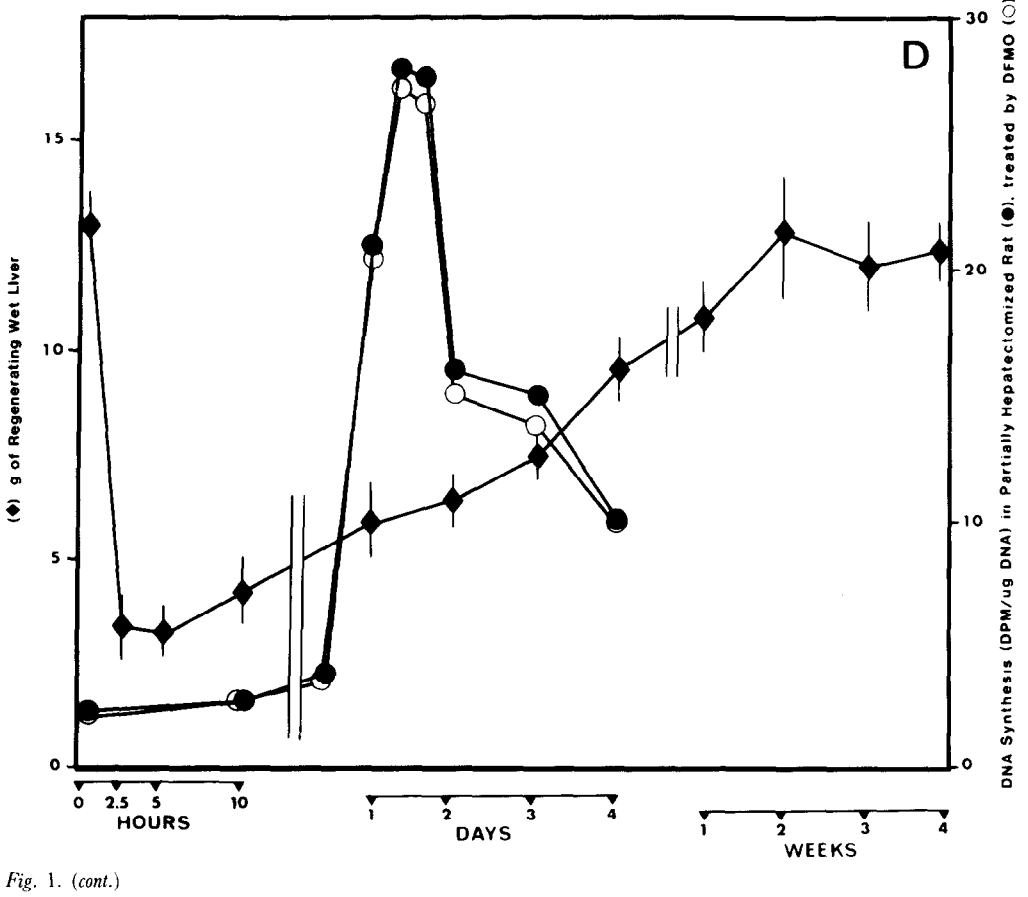

D
(◆) g of Regenerating Wet Liver
DNA Synthesis (DPM/ug DNA) in Partially Hepatectomized Rat (●), treated by DFMO (○).
HOURS
DAYS
WEEKS

*Fig.* 1. (*cont.*)

*Table 1. Red blood cell (RBC) and liver polyamine levels in sham-operated and in one-third partially hepatectomized rats*

| Time intervals (hr) | | Red blood cell | | | Liver | | |
|---|---|---|---|---|---|---|---|
| | | Putrescine | Spermidine (nmol/8.10⁹ RBC) | Spermine | Putrescine | Spermidine (nmol:g tissue) | Spermine |
| 0 hr | Controls | 1.70±0.27 | 10.53±3.64 | 3.14±0.18 | 5.00±0.25 | 319.02±17.09 | 441.14±53.08 |
| 2 hr 30 | Sham-operated | 3.58±0.70[a] | 25.60±4.37[a] | 2.19±0.24 | 16.44±5.02[a] | 280 ±39.90 | 416.25±42.35 |
| 2 hr 30 | Regenerating | 3.04±0.38[a] | 34.98±4.06[b] | 2.35±4.06 | 16.55±4.82[a] | 340.20±32.30 | 400.10±56.86 |
| 5 hr | Sham-operated | 2.62±0.30 | 15.43±3.89 | 2.56±1.15 | 52.21±8.50[b] | 389.07±29.28 | 463.75±29.66 |
| 5 hr | Regenerating | 3.45±0.54[a] | 22.05±1.26*[a] | 2.79±0.53 | 60.31±9.86[b] | 455.12±33.20 | 450.62±49.42 |
| 10 hr | Sham-operated | 2.29±0.20 | 13.90±0.74 | 1.60±0.32 | 13.20±5.23[a] | 300.48±38.86 | 462.76±65.52 |
| 10 hr | Regenerating | 3.18±0.69a | 20.01±4.64*[a] | 1.49±0.25 | 15.17±4.24[a] | 485.72±40.70* | 440.80±36.20 |
| 24 hr | Sham-operated | 2.52±0.27 | 13.59±6.11 | 2.25±0.47 | 4.61±0.68 | 333.58±29.60 | 422.19±31.74 |
| 24 hr | Regenerating | 4.42±0.94b | 28.44±9.60**[b] | 2.99±0.75 | 10.36±5.67 | 610.65±30.55**[a] | 426.82±29.60 |
| 48 hr | Sham-operated | 2.66±0.31 | 12.02±2.08 | 1.59±0.21 | 5.60±0.25 | 329.02±17.90 | 461.14±53.08 |
| 48 hr | Regenerating | 3.08±0.54[a] | 15.88±2.74 | 2.35±1.45 | 8.92±6.56 | 420.30±28.72* | 418.24±40.72 |

(*) Differs from sham-operated controls ($P < 0.01$).
(**) Differs from sham-operated controls ($P < 0.001$).
Results as shown as mean ± S.D. [a] Differs from controls ($P < 0.01$); [b] Differs from controls $P < 0.001$).
Groups of five rats were sham-operated or one-third partially hepatectomized and killed at varying intervals. RBC and livers were assayed for levels of putrescine, spermidine and spermine.

*Table 2. Picomoles of polyamines incorporated by 4.10⁹ RBC incubated during 1 hr in the presence of homologous serum at 37° C with 2 nmol each of [¹⁴C]-putrescine, [¹⁴C]-spermidine or [¹⁴C]-spermine, and increasing amounts of αDFMO*

| Concentrations of αDFMO | Putrescine (pmol of ¹⁴C polyamine/4.10⁹ RBC) | Spermidine | Spermine |
|---|---|---|---|
| 0 | 171.4 | 223.4 | 50.60 |
| 0.5 mM | 152.5 (N.S) | 194.35* | 39.47* |
| 2.5 mM | 92.5* | 140.74** | 26.32** |
| 5.0 mM | 53.1** | 93.83*** | 26.32*** |

Values are expressed as means of four triplicate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, statistically significant differences in comparison with controls.

[11] that the diminution of [³H]-thymidine incorporation in the liver of partially hepatectomized rats receiving a high dose of α-DFMO appears correlated to liver spermidine accumulation.

Moreover, these authors have also observed that only 30–40% of the total putrescine synthesized during the course of liver regeneration are needed for the enhanced DNA synthesis following partial hepatectomy [11]. The rise of RBC spermidine levels observed after a two-third partial hepatectomy could in part explain the results obtained by Poso and Pegg in α-DFMO treated animals. Under our experimental conditions, we did notice that, though α-DFMO modified neither [³H]-thymidine incorporation, nor spermidine and spermine hepatic concentrations, this drug lowered levels both of liver putrescine and RBC spermidine. Thus an excess of liver spermidine produced from an excess of putrescine synthesized during the first hours following a partial hepatectomy could be released in blood and taken up by erythrocytes, especially as spermidine affinity is at least 30 times higher than that of putrescine for RBC [18]. It is thus quite likely that α-DFMO could reduce both putrescine and spermidine concentrations in regenerating liver, but the decrease of this hepatic polyamine level would only relate to the excess of free spermidine usually taken up by erythrocytes.

From the fifth hour to the tenth after a two-third partial hepatectomy, RBC spermidine levels become similar to those of α-DFMO treated animals (Fig. 1B): *in vivo*, the spermidine half-life in erythrocytes could be estimated to 2.5–3.0 hr, which could explain the correlated elevations of hepatic and RBC spermidine levels from the tenth hour to the fourth week of regeneration.

The elevation of the erythrocyte spermidine concentration not being dependent on the regenerating liver weight, RBC spermidine levels rather appear to be related to the hyperplastic period of liver regeneration. Those results are corroborated by the fact that a one-third partial hepatectomy is responsible for liver and erythrocyte spermidine levels in proportion to the hepatic parenchyma cutting away, and erythrocyte spermidine levels, though not directly involved in the hyperplastic mechanism, seem to reflect the cell-proliferating process.

This particular relationship between tissue and erythrocyte spermidine concentrations deserves clinical attention: as is the case for the Lewis tumor and liver regeneration, RBC spermidine levels in non-treated animals are correlated to those of tissue spermidine, the determination of erythrocyte

spermidine concentrations in patients harboring solid tumors would enable determination of the intratumoral hyperplastic level. In the case of patients suffering from bronchopulmonary cancers [20] or from intracranial [19] and malignant hepatic [18] tumors we have indeed observed a link between the histological grade of malignancy and the value of RBC spermidine level. If we admit that a high grade of malignancy can be explained in terms of rapid cell proliferation, the evaluation of RBC spermidine concentrations which seemed linked to cellular proliferating activity, could contribute to determining the level of intratumoral proliferation in cancerous patients. Thanks to this possible evaluation of the level of intratumoral proliferation, patients could be followed up and treated according to that parameter.

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