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DIFFERENTIAL RESPONSE OF MITOCHONDRIAL AND NUCLEAR DNA SYNTHESES TO HYDROXYUREA IN NORMAL AND REGENERATING RAT LIVER

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Abstract—Male rats were injected with hydroxyurea (100 mg/kg or 500 mg/kg) and sacrificed at different times after injection. [^{14}C]-thymidine is injected 1 hr before sacrifice and the specific radioactivity determined in total DNA of spleen, thymus and regenerating liver and in nuclear and mitochondrial DNA of normal and regenerating liver. Total DNA synthesis is rapidly inhibited in lymphoid tissues and in regenerating liver, but recovery is more rapid in the latter organ. Inhibition of DNA synthesis occurs more slowly in the mitochondria than in the nuclei of normal and regenerating liver; the rate of recovery is also lower in the mitochondria. These differences between the two DNA are tentatively ascribed to a less rapid exhaustion of the intra-mitochondrial pool of deoxynucleotides as regards the inhibition phase, and to a competition for the precursor pool between N-DNA and M-DNA syntheses, in favor of the predominant one (N-DNA synthesis) as regards the recovery phase.

THE CHEMOTHERAPEUTIC agent hydroxyurea (HOU) is known as a selective inhibitor of DNA synthesis in bacterial as well as in mammalian cells, without interfering with RNA and protein syntheses.[1–6]

One of the primary enzymatic sites of action of the drug in the DNA metabolism appears to be localized at the level of the ribonucleotide reductase.[6–8]

In continuously dividing tissues (intestinal epithelium, lymphoid tissues), the inhibition of DNA synthesis is associated with extensive necrosis, whereas the inhibition also observed in tissues with induced mitotic activity, such as regenerating liver, is not associated with cell death.[9,10]

On the other hand, it is now well established that mitochondrial (M-) and nuclear (N-) DNA syntheses display striking differences in their structural and biochemical properties (see refs. 11, 12); the structure of M-DNA appears to be quite similar to that of bacterial DNA. Moreover, in a resting tissue such as normal rat liver, the rate of M-DNA synthesis has been shown to be much higher than that of N-DNA synthesis. In regenerating liver, the N-DNA synthesis remains at a low level during the first 12 hr after partial hepatectomy and then increases rapidly until the 24th hr, whereas the stimulation of M-DNA synthesis is low, but almost linear from the time of partial hepatectomy.[13]

The purpose of the present work was mainly to follow the kinetics of response of these two DNA syntheses to the administration of hydroxyurea in normal and

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regenerating rat liver, in order to compare the degree of dependency of both syntheses on the synthesis of precursors.

MATERIALS AND METHODS

Male Wistar rats, (180–200 g) were used throughout. Partial hepatectomies were performed according to the technique of Higgins and Anderson.[14] The rats were fasted 24 hr before sacrifice and killed by decapitation between 9.30 and 11.30 a.m.

Groups of eight rats received, at the indicated times, one single intraperitoneal injection of 100 or 500 mg/kg of hydroxyurea, dissolved in 1 ml 0·14 M NaCl. Corresponding groups of controls, injected with 0·14 M NaCl, were always included.

One hr before sacrifice, the rats were injected intravenously with 10 μc of [^{14}C]-thymidine (specific radioactivity: 48 mc/m-mole).

Preparation and fractionation of the livers

After sacrifice, the livers were quickly excised and perfused with ice-cold 0·25 M sucrose; the livers from eight rats were pooled, minced with scissors and homogenized in 7 vols. of 0·25 M sucrose, in a Potter tube fitted with a teflon pestle. The homogenate was centrifuged for 10 min at 600 *g* to sediment the nuclear fraction. The supernatant was submitted to a second centrifugation at 800 *g* for 10 min: the supernatant (cytoplasmic fraction) was carefully removed and the pellet added to the nuclear fraction.

(a) *Isolation of the nuclei.* The purification of the nuclei in 2·2 M sucrose was performed according to the technique of Chauveau *et al.*[15] The nuclear pellet was homogenized with 10–15 vols. of 2·2 M sucrose and the suspension was spun for 60 min at 40,000 *g*. The supernatant was discarded and the nuclei were washed twice by resedimentation from 0·25 M sucrose and suspended in 20 ml 0·15 M NaCl, 0·1 M EDTA pH 8·0.

(b) *Preparation of the mitochondria.* The cytoplasmic fraction was centrifuged at 7500 *g* for 10 min and the supernatant discarded. The mitochondria were washed twice by resedimentation from 0·25 M sucrose and once by resedimentation from 0·15 M NaCl and incubated for 30 min at 37° with DNase I (20 μg/ml) and $MgCl_2$ (0·005 M) to remove residual nuclear DNA.[16] DNase digestion was stopped by adding 1 vol. ice-cold 0·15 M NaCl, 0·1 M EDTA pH 8·0 and the DNase was removed by recentrifugation from the same medium. Finally, the mitochondrial pellet was suspended in 20 ml 0·15 M NaCl, 0·1 M EDTA pH 8·0.

The efficiency of the DNase treatment has been controlled in a way similar to that described by Nass:[16] two samples of the same preparation of mitochondria were processed according to the technique described here, except that, before DNase digestion, one sample was added with a suspension of nuclei, the proportion of N-DNA being about 20 times that of M-DNA. After extraction, the amount of DNA and the specific radioactivity detected in both samples were in good agreement (deviation < 2 per cent).

When the DNAs are not labelled, the determination of equilibrium density in CsCl or sedimentation velocity may be required to distinguish between N-DNA and M-DNA. But since M-DNA of normal rat liver incorporates so much more thymidine than N-DNA so as to have a specific radioactivity 23 times greater than that of N-DNA in our experimental conditions,[17] the presence of a high specific radioactivity

is in itself a sensitive indicator of the purity of the M-DNA. The persistence of the same high specific radioactivity of M-DNA even after addition of 20 times its amount of N-DNA followed by DNase digestion, as described above, proves the validity of the method used and points to a complete removal of any contaminating N-DNA in mitochondrial preparations incubated with DNase.

Further purification of mitochondria by centrifugation in a linear sucrose gradient (1·04–1·8 M sucrose) led to results which did not differ significantly (< 5 per cent) from those obtained by the technique described above. Therefore, the sucrose gradients were not used routinely.

Preparation of spleen and thymus extracts

After sacrifice, the spleens and thymus were quickly excised, dropped into flasks immersed in acetone–dry ice and stored at −30° until processed. The organs of each group of eight rats were pooled and homogenized in 7–10 vols. 0·15 M NaCl, 0·1 M EDTA pH 8·0, by means of a Potter tube fitted with a teflon pestle.

Extraction and measure of the specific radioactivity of DNA

One ml of 25 per cent aqueous solution of sodium dodecylsulfate (SDS) was added to each 14 ml of tissue extract. The mixture was heated for 10 min at 60°, cooled in an ice bath and the DNA was extracted according to the procedure of Schmidt–Tannhauser (see Hutchinson and Munro[18]). The amount of DNA was quantitatively determined by the diphenylamine reagent[19] using calf thymus DNA as reference. The radioactivity of the DNA was measured by liquid scintillation in a Packard Tri-Carb counter.

The validity of this extraction procedure has been tested by comparison with a more elaborated technique: after treatment with SDS, the organelles were incubated with pronase (1 mg/ml) for 60 min at 37°. The suspension was then deproteinized by shaking for 30 min at room temperature with an equal volume of chloroform–isoamyl alcohol (24:1) and centrifuged.[20] The aqueous phase was again shaken with chloroform–isoamyl alcohol and centrifuged. The nucleic acids were precipitated by adding 2 vols. ethanol and storing the mixture in the cold room overnight.

The nucleates were then sedimented and redissolved in 0·15 M NaCl. After precipitation by addition of $HClO_4$ to a final concentration of 0·6 N, the extracts were then submitted to the Schmidt–Tannhauser procedure as above. The values obtained by these two techniques were in excellent agreement (deviation ≤ 3 per cent).

RESULTS

(1) The kinetics of the inhibition of DNA synthesis has been studied in various rat tissues. A single injection of HOU was given at different times (1–12 hr) before sacrifice and the rate of DNA synthesis was measured by the incorporation of [^{14}C]-thymidine into the DNA.

As seen in Fig. 1 (left), both doses of HOU (100 or 500 mg/kg) produced a rapid and nearly complete inhibition of DNA synthesis in lymphoid tissues such as spleen and thymus. The duration of the inhibition and the rate of recovery depended on the dose.

In a resting tissue, the normal rat liver (Fig. 1, right), where the DNA synthesis proceeds slowly, the specific radioactivity of DNA decreased less rapidly than in

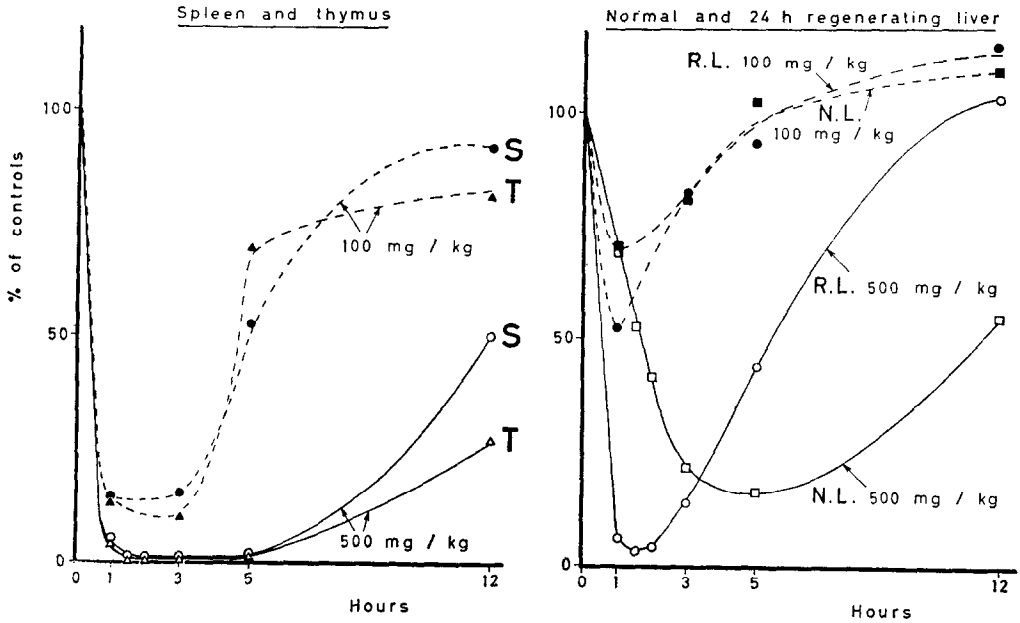

FIG. 1. Total DNA synthesis in various rat tissues, at different times after one intraperitoneal injection of hydroxyurea. 10 μc of [^{14}C]-thymidine are always injected intravenously 1 hr before sacrifice. Left: spleen (S) and thymus (T). Right: normal (N.L.) and 24 hr regenerating liver (R.L.). Ordinates: specific radioactivity in per cent of control values. Abscissae: time-interval between injection of HOU and sacrifice. Full lines (○): 500 mg/kg HOU. Dotted lines (●): 100 mg/kg HOU.

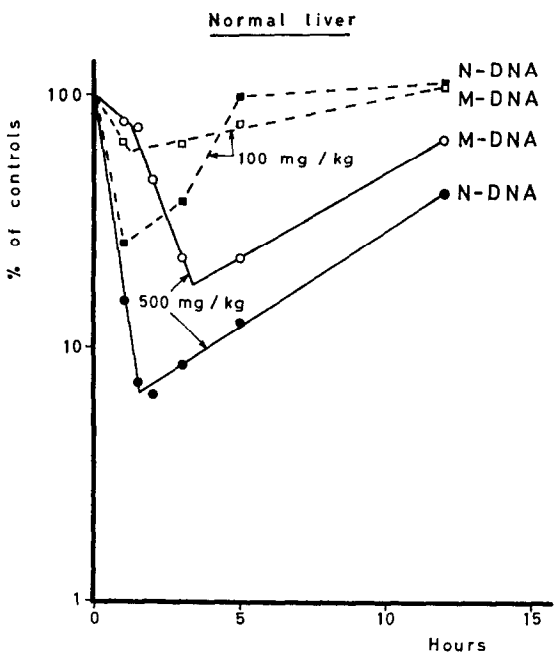

FIG. 2. Incorporation of [^{14}C]-thymidine into the M-DNA and the N-DNA of normal rat liver, after one i.p. injection of HOU. Ordinates and abscissae; see Fig. 1. Open symbols: M-DNA; closed symbols: N-DNA. Full lines (○): 500 mg/kg HOU; dotted lines (□): 100 mg/kg HOU.

normally proliferating tissues: a dose of 100 mg/kg produced an inhibition of only 30 per cent, 1 hr after injection and then the specific radioactivity resumed to a normal level, reached at the 5th hour. The dose of 500 mg/kg produced a maximal inhibitory effect between the 3rd and 5th hour and recovered to 50 per cent of the controls when the lapse of time between injection and sacrifice was 12 hr. In 24 hr regenerating liver, the rate of DNA synthesis showed a sharp decline, with a maximum of inhibition at 1–2 hr, similar to spleen and thymus, but the *extent* of maximal inhibition was more closely related to the dose in the case of regenerating liver and the *recovery* was more complete than in lymphoid tissues, 12 hr after a dose of 500 mg/kg.

(2) The response of mitochondrial and nuclear DNA syntheses to the administration of hydroxyurea has been analyzed in normal and in regenerating rat liver (Figs. 2–4).

In all cases, it is clear that the incorporation of labelled thymidine into M-DNA was less sensitive to the effects of the drug than the incorporation of the precursor into the N-DNA.

In normal liver (Fig. 2), the maximal inhibition of N-DNA synthesis (94 per cent) was reached between 1 and 2 hr after injection of 500 mg/kg HOU, whereas the maximal inhibition of M-DNA synthesis (80 per cent) was reached only between 3 and 5 hr after injection, but the rate of the exponential recovery of both DNAs as a function of the time was similar. To a dose of 100 mg/kg, the M-DNA still responded in an

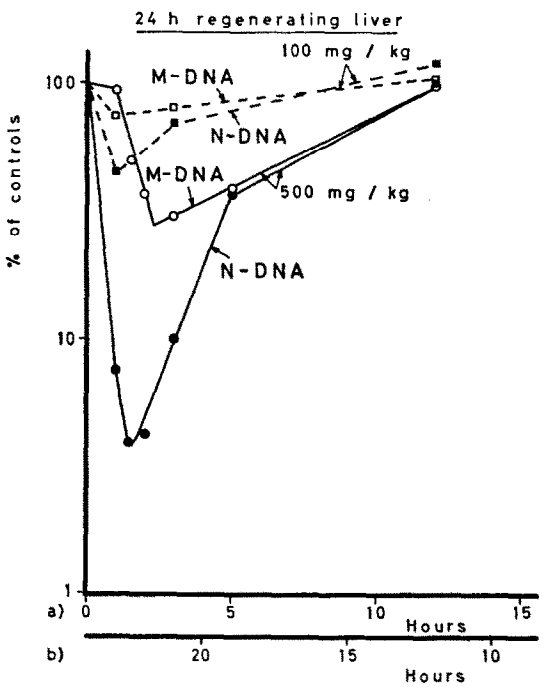

FIG. 3. Incorporation of [^{14}C]-thymidine into M- and N-DNA of 24 hr regenerating liver, after injection of HOU. Ordinates: specific radioactivity, in per cent of control values. Abscissae: (a) time-interval between injection and sacrifice. (b) corresponding time of liver regeneration: partial hepatectomy at Oh. Symbols and lines as in Fig. 2.

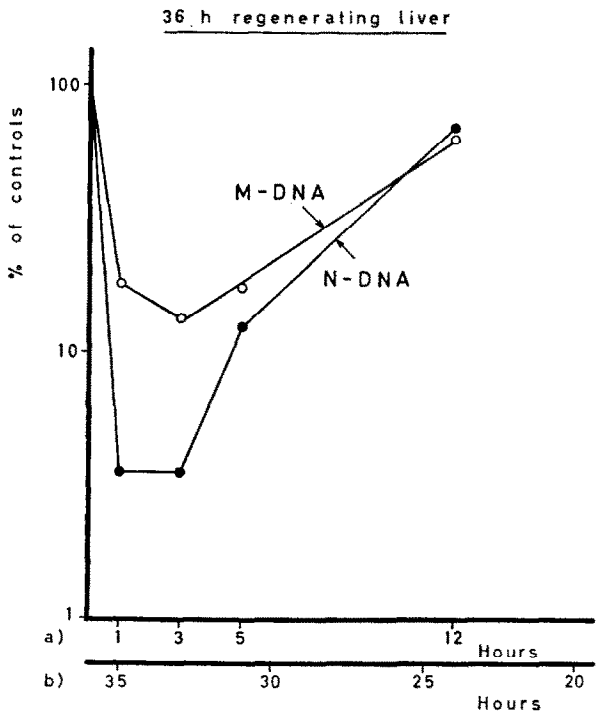

FIG. 4. Incorporation of [^{14}C]-thymidine into M- and N-DNA of 36 hr regenerating liver, after injection of 500 mg/kg HOU. Ordinates and abscissae: see Fig. 3. Open symbols: M-DNA. Closed symbols: N-DNA.

exponential way, but no parallelism was observed between M- and N-DNA any more, the N-DNA synthesis resuming to the control values earlier than the M-DNA synthesis.

Figure 3 shows that in 24 hr regenerating liver, contrary to the normal liver, a complete recovery of both N- and M-DNA syntheses was obtained at the 12th hr, even after a dose of 500 mg/kg. Again, the response of M-DNA to 100 or 500 mg/kg of HOU was exponential, characteristic of a continuously synthesizing system. The time required to reach the maximum of inhibition and the magnitude of this inhibition are directly related to the dose. As in all the other cases, the effect on N-DNA synthesis is certainly related to the actual concentration of the drug in the tissue, but if one considers the slope of the curves, the recovery appears to be much faster from the 2nd to the 5th hr than from the 5th to the 12th hr. This must probably be correlated with the kinetics of the N-DNA synthesis during the first mitotic cycle after partial hepatectomy.

Some results obtained with 36 hr regenerating liver are presented in Fig. 4. They confirm the data obtained with 24 hr regenerating liver. The same pattern of exponential recovery was observed in the case of M-DNA synthesis, with a response somewhat more complex for N-DNA synthesis.

DISCUSSION

(1) In all proliferating tissues, hydroxyurea produces a rapid and dose-dependent inhibition of DNA synthesis, in agreement with previous observations.[5,6,9] This is

well illustrated in Fig. 1 for spleen, thymus and regenerating liver. But whereas the incorporation rate has resumed to normal values after 12 hr in regenerating liver, it remains at low levels (50 and 26 per cent respectively) in spleen and thymus, in animals injected with 500 mg/kg of HOU. The occurence of cell necrosis in lymphoid tissues of rats treated with HOU can most probably account for the much slower recovery of DNA synthesis in spleen and thymus, in comparison to regenerating liver which does not show any cell necrosis after HOU.[10] Farber and Baserga indeed observed a modest central necrosis of the germinal centers of lymph follicles 3·5 hr after injection of 150 mg/kg HOU. The dose of 500 mg/kg used here certainly produces a non negligible necrosis whereas the dose of 100 mg/kg allows a very significant recovery to take place (Fig. 1).

The inhibition and the recovery of DNA synthesis both occur more rapidly in 24 hr regenerating liver than in normal liver after injection of 500 mg/kg HOU (Fig. 1, right). Since HOU mainly acts by inhibiting the ribonucleotide reductase,[6–8] its inhibitory power will depend on the ratio inhibitor/enzyme. Because the activity of the reductase markedly increases from the 18th hr after partial hepatectomy,[8] the ratio inhibitor/enzyme will be increasingly smaller in regenerating liver compared to normal liver and the inhibition, therefore, more easily reversible in the former organ, as shown in Fig. 1. The fact that inhibition of DNA synthesis also develops more rapidly in regenerating liver than in normal liver suggests that the supply of reduced nucleotides is much more quickly exhausted in 24 hr regenerating liver which indeed synthesizes DNA several hundred fold more rapidly than normal liver.[21]

(2) The study of M- and N-DNA syntheses provides the opportunity to compare two DNAs, very different by most of their properties and present simultaneously in the same organ.

It appears from the results presented in Figs. 2–4, that the incorporation of [^{14}C]-thymidine into M-DNA is less inhibited by the injection of hydroxyurea than the incorporation of the precursor into the N-DNA. This interesting property is shared by the cytoplasmic DNA from HeLa cells cultured in presence of hydroxyurea.[22]

The interpretation of the present results must take into account the differential behavior of M- and N-DNA in normal and regenerating rat liver.[13] In the normal rat liver, the M-DNA synthesis proceeds at a much faster rate than the N-DNA synthesis. As shown in Fig. 2, the extent and the rate of inhibition are not directly related to the rate of synthesis; indeed, in the case of M-DNA, the maximal inhibition (80 per cent) is reached between 3 and 5 hr after injection, whereas in N-DNA, the maximal inhibition (94 per cent) is already obtained 1–2 hr after injection of HOU. This situation is therefore quite different from what was observed by comparing total DNA synthesis of different organs (Fig. 1). Whereas the rate of inhibition by HOU was directly related to the initial rate of synthesis in the case of the various DNAs, the relationship is reversed when M-DNA is compared to N-DNA in normal liver. Of course, in normal rat liver, few cells synthesize N-DNA (1/20,000) whereas many more cells are engaged in M-DNA synthesis. In many cells, therefore, the whole supply of deoxynucleotides is at the disposal of M-DNA, but in few cells, competition will occur between N- and M-DNA syntheses for the common pool of precursors.

Nothing is known about the pool of deoxyribonucleotides in the mitochondria. Several reasons render rather unlikely the possibility of a conversion of ribonucleotides into deoxyribonucleotides inside the mitochondria: they are excellently reviewed by

Borst and Kroon[23] and are also strengthened by the more recent observation of Larsson[24] that, after fractionation by differential centrifugation, only trace activity of ribonucleotide reductase can be found in regenerating liver mitochondria, and no activity at all in normal liver mitochondria. Therefore the penetration of HOU inside the mitochondria is of minor importance since the target-enzyme is cytoplasmatic. In the cells engaged in both N-DNA and M-DNA synthesis, since mitochondrial DNA only represents 2–3 per cent of the total cell DNA, the drain on the precursor pool will be much less by the limited DNA synthesis in the mitochondria as compared to that in the nucleus. The available precursor pool is therefore likely to be less rapidly exhausted in mitochondria than in the nuclei. Figure 2 shows indeed that the inhibition of M-DNA synthesis develops more slowly than that of N-DNA. Competition for the precursor pool between N-DNA and M-DNA synthesis, in favor of the predominant one—N-DNA synthesis—might account for the observation that M-DNA synthesis recovers more slowly than N-DNA synthesis, at least after administration of the smaller dose of HOU (100 mg/kg) (Fig. 2).

The greater rate of recovery of N-DNA synthesis compared to that of M-DNA synthesis is particularly noticeable in 24 hr regenerating liver (Fig. 3). At the time when the recovery begins, at the 22nd hr of regeneration (see Fig. 3), ribonucleotide reductase activity is normally very much increased so that reversion of the inhibition will lead to a rapid formation of the precursors. Moreover, it has been found that in several cell types, addition of HOU to tissue cultures produces an increase in deoxynucleotide kinase activity, due to unbalanced growth.[25] If this effect also occurs in regenerating liver after HOU treatment, it would undoubtedly speed up the rate of recovery, as does the presence of an increased amount of ribonucleotide reductase.

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