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Overproduction of the free radical of ribonucleotide reductase in hydroxyurea-resistant mouse fibroblast 3T6 cells

(subunit structure/enzyme regulation/deoxynucleoside triphosphate pools/EPR)

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ABSTRACT **Hydroxyurea inhibits the activity of ribonucleotide reductase (ribonucleoside-diphosphate reductase; 2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1) in bacteria and mammalian cells. The reductase from *Escherichia coli* consists of two nonidentical subunits (B1 and B2) and hydroxyurea acts by specifically destroying a tyrosine free radical of B2 required for enzyme activity. The mammalian enzyme also consists of two nonidentical subunits (M1 and M2), only one of which (M1) has been obtained in pure form. By continuous culture at stepwise increasing drug concentrations, we have now obtained a 3T6 mouse fibroblast cell line with a 100-fold increased resistance to hydroxyurea. Extracts from resistant cells showed a 3- to 15-fold increase in reductase activity. The amount of M1 protein was not increased. The amount of M2 protein could not be measured directly, but the M2 activity in extracts from resistant cells was increased 27-fold. Furthermore, the packed resistant cells (but not normal cells) showed an EPR spectrum very similar to that of the tyrosine radical of the bacterial B2 subunit. We propose that resistance to hydroxyurea is caused either by overproduction of the complete M2 subunit or by increased generation of the tyrosine radical within the M2 protein. It seems that either alternative mirrors a possible normal regulatory mechanism for the activity of the reductase.**

In bacteria as well as in mammalian cells hydroxyurea inhibits DNA synthesis by interaction with the enzyme ribonucleotide reductase (ribonucleoside-diphosphate reductase; 2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1) (for review see ref. 1). In *Escherichia coli* this enzyme consists of two nonidentical subunits, called proteins B1 and B2. The B2 subunit contains iron and a tyrosine free radical characterized by a specific EPR signal and essential for enzyme activity. Incubation of protein B2 with hydroxyurea irreversibly destroys the radical and the enzyme activity.

The best-characterized mammalian reductase is the enzyme from calf thymus (2). This enzyme also appears to consist of two subunits, proteins M1 and M2 (3). Only the M1 subunit has so far been obtained in homogeneous form; it appears in many respects to be equivalent to the bacterial B1 subunit. The properties of M2 are poorly characterized because of limited purification. M2 appears to contain iron but no evidence for a free radical has as yet been obtained. Hydroxyurea inhibits the thymus enzyme in a reversible fashion.

In this communication we report the results from experiments with 3T6 mouse fibroblasts with an up to 100-fold increase in resistance to hydroxyurea. Resistant cell lines were obtained by growing the parent line over a period of 2 years in the presence of stepwise increasing concentrations of hydroxyurea. In these experiments we were guided by similar earlier experiments in which resistant cell lines were obtained by continuous culture with amethopterin or *N*-phosphonacetyl-L-aspartate and shown to overproduce the respective target enzymes dihydrofolate reductase (4) and aspartate carbamoyltransferase (5). Resistance to hydroxyurea has been reported to be caused either by a change in the sensitivity of ribonucleotide reductase or by overproduction of the enzyme (6).

Extracts from our resistant cells showed an about 10-fold increase of reductase activity and a 30-fold increase in M2 activity. The M1 protein was not overproduced. EPR spectroscopy of packed resistant cells revealed a signal very similar to that characterizing the tyrosine radical of the bacterial B2 subunit. This signal was not detected in the parent 3T6 line.

MATERIALS AND METHODS

Materials. Radioactive cytidine, nucleotides, amino acids, and ^{125}I-labeled Bolton and Hunter reagent for iodination of staphylococcal protein A (Pharmacia) were obtained from the Radiochemical Centre, Amersham. DNA polymerase I from *E. coli* was a gift from G. Magnusson of the Karolinska Institute, and poly[d(A-T)] and poly[d(I-C)] were purchased from Boehringer. Hydroxyurea was obtained from Sigma. Homogeneous protein M1 from calf thymus was prepared as described (3).

Cell Lines. The derivation and some of the characteristics of the hydroxyurea resistant cell lines will be described elsewhere. Briefly, 3T6 cells were grown over a period of about 2 years at increasing (0.1–4 mM) concentrations of hydroxyurea. At different stages the resistance of the cell populations was tested by measuring plating efficiency or cell growth (7) during 72 hr at different concentrations of hydroxyurea. With the original 3T6 cells, 50% inhibition of plating efficiency was observed at 0.05 mM hydroxyurea, whereas the cloned HU-1 and HU-11 lines showed a similar inhibition at 1 and 6 mM, respectively. Line HU-11R, which was obtained from HU-11 by 40 continuous passages in the absence of the drug, was inhibited 50% by 2 mM hydroxyurea.

Cell Cultures. Monolayer cultures were maintained in Dulbecco's modification of Eagle's medium plus 10% fetal calf serum or 10% heat-inactivated horse serum.

Enzyme Assay. Ribonucleotide reductase activity was determined by measuring the reduction of [^{3}H]CDP as described (2). One unit of ribonucleotide reductase activity is defined as the amount of enzyme, or subunit in the presence of the other subunit, that catalyzes the formation of 1 nmol of deoxycytidine diphosphate per min at 37°C.

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Protein Determination. Protein was determined according to Lowry as modified by Jovin by using bovine serum albumin as a standard or by the Coomassie brilliant blue G-250 method calibrated by the Lowry method (cf. ref. 2).

Preparation of Cell Extracts. Cells were taken in the early logarithmic phase of growth and the medium was removed. Each 14-cm dish was washed twice with 25 ml of ice-cold Tris/saline and once with 20 ml of ice-cold 20 mM 4-(2-hydroxyethyl)-1-piperazinesulfonic acid buffer, pH 7.5/10 mM $MgCl_2$/2 mM dithiothreitol. The dishes were left in a vertical position at 4°C for 10 min, residual buffer was removed, and then the cells were scraped from the dishes. The cell suspension was tranferred to a glass homogenizer and after homogenization the suspension was cleared by centrifugation in a Sorval SS-34 rotor at 15,000 rpm for 30 min at 0°C. The solution, containing 2–5 mg of protein per ml, was rapidly frozen and kept at −70°C.

Pulse Labeling of Cells. Parent and resistant cells were grown in a leucine-free medium and in the early logarithmic phase they were pulsed for 4 hr with [^{14}C]leucine (25 μCi/10 ml of medium, specific activity 330 mCi/mmol) and [^{3}H]leucine (60 μCi/10 ml of medium, specific activity 800 mCi/mmol), respectively (1 Ci = 3.7×10^{10} becquerels). At the end of the pulse 70% of the radioactivity had been taken up by the cells. Cell extracts were prepared as described above.

Preparation of Cells for the EPR Measurements. The medium was removed from parent or resistant cells in the early logarithmic phase and the dishes were washed with Tris/saline. After trypsin treatment, the cells were suspended in medium containing 10% serum, centrifuged, washed once in Tris/saline, and again collected by centrifugation. The cell suspension was finally transferred directly to an EPR tube, packed by centrifugation, frozen, and stored in liquid nitrogen. In each experiment a 150 μl of cell suspension containing 7.7×10^7 cells was used.

EPR Measurements. First-derivative EPR spectra were recorded on a modified Varian V-4502 spectrometer as described (8).

RESULTS

Sensitivity to Hydroxyurea of DNA and dCTP Synthesis in 3T6 and Resistant Cells. The effects of different concentrations of hydroxyurea on the incorporation of labeled cytidine into 3T6 cells and various resistant cell lines are shown in Fig. 1. Under the conditions of our experiment incorporation of cytidine into dCTP and DNA measures *in situ* ribonucleotide reduction and DNA synthesis, respectively (unpublished data). The results demonstrate a parallel inhibition by hydroxyurea of both types of incorporation and of cell growth.

Increased Activity of Ribonucleotide Reductase in Resistant Cells. Assaying ribonucleotide reductase in crude cell extracts is fraught with considerable difficulties because of large variations during the cell cycle and because of the complicated allosteric regulation of the enzyme (9). However, in many experiments, either with cell extracts or permeabilized cells, we consistantly found a 3- to 15-fold higher enzyme activity in resistant cells than in the parent line. In some of these experiments the cell extracts were treated with Dowex-1 Cl^- in order to avoid inhibitory effects of dNTPs. Variations during the growth cycle are illustrated in Fig. 2. Both parent and resistant cells showed the highest activities on the second day after explantation, when a majority of cells was involved in DNA synthesis. At this point extracts from the resistant cells contained 10 times more enzyme activity. In both lines enzyme activity was barely detectable when DNA synthesis had stopped (as judged from autoradiography). When DNA synthesis of the resistant cells was stimulated in the middle of the growth curve by addition of fresh medium, a second peak of enzyme activity occurred (Fig. 2).

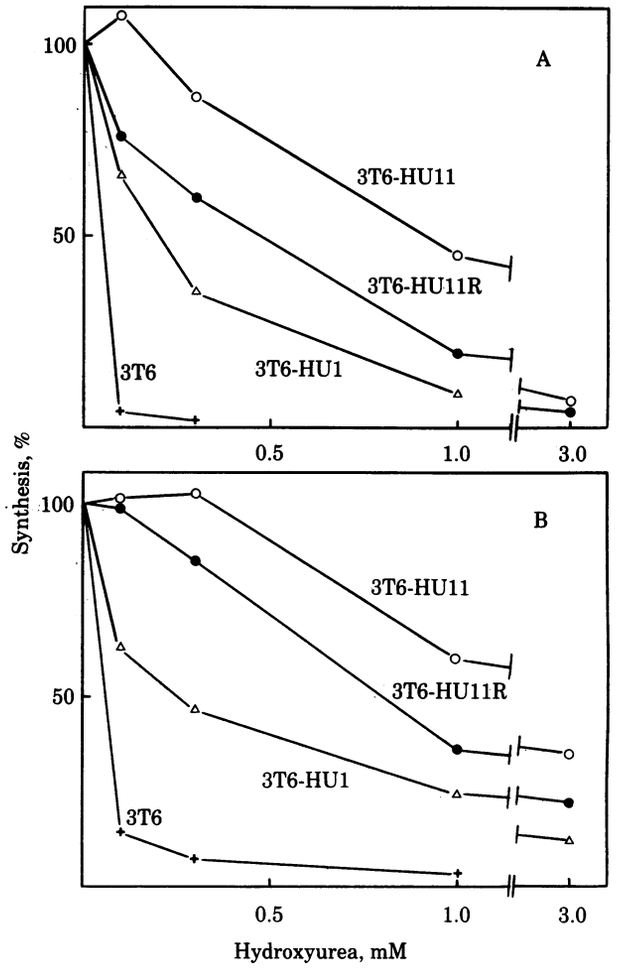

FIG. 1. Incorporation of cytidine into DNA (*A*) and dCTP (*B*) in parent and resistant cell lines. Growing cells on 3-cm dishes were incubated for 60 min with 3 μM [^{3}H]cytidine (6000 cpm/pmol) and different concentrations of hydroxyurea, washed three times with ice-cold Tris/saline, and extracted with 1 ml of ice-cold 1 M $HClO_4$. The residue was dissolved in 1 ml of 0.4 M NaOH and incubated overnight at 37°C, and aliquots were used for the determination of acid-precipitable (= DNA) radioactivity. The perchloric acid extracts were heated at 100°C for 10 min and neutralized with 4 M KOH, and the supernatant solutions after centrifugation were chromatographed on Dowex-50 to separate CMP and dCMP. The radioactivity in DNA and dCMP, respectively, from the noninhibited cultures is taken as 100%.

The reductase activity in both types of extracts showed a similar sensitivity towards inhibition by different concentrations of hydroxyurea (data not shown).

Differences in dNTP Pools. The increased reductase activity in resistant cells might be reflected in differences in dNTP pools between 3T6 and resistant cells. When we analyzed the size of all four dNTP pools during growth of the two cell lines, in both cases we observed the largest pool sizes early during growth, when DNA synthesis was most active (Fig. 3). In 3T6 cells at that point the two pyrimidine pools dominated, with dCTP being the largest pool. dATP occupied an intermediate position and dGTP had the smallest pool. On prolonged growth the size of all four pools decreased, the two pyrimidines more than dATP.

The most pronounced difference in the resistant cells concerned the dATP pool which was increased 3-4-fold in size compared to 3T6 cells. It exceeded at all time points the dCTP pool

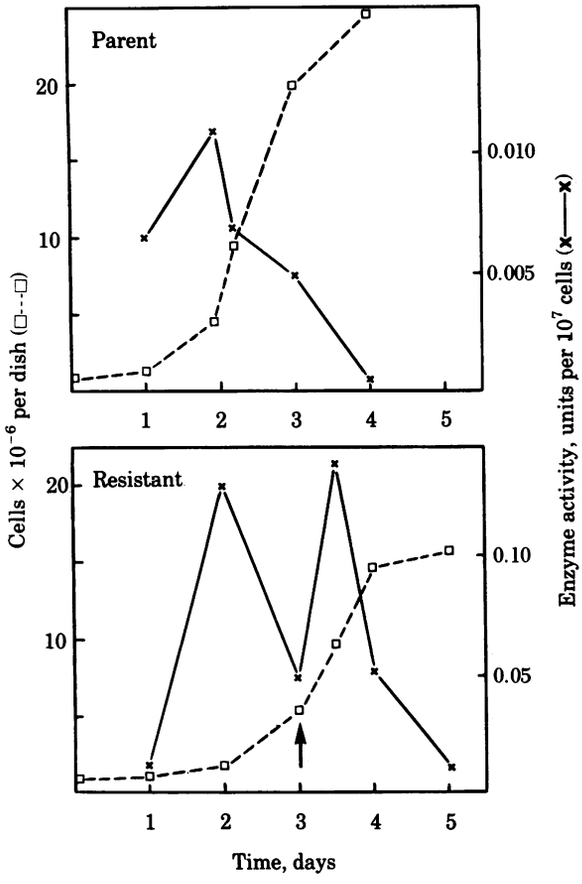

FIG. 2. Ribonucleotide reductase activity in parent (*Upper*) and hydroxyurea-resistant cells (*Lower*). Cells were grown in the absence of hydroxyurea, and 3 days after explantation (arrow) fresh medium was added to the resistant cells. The cells were harvested by trypsinization and counted, and after permeabilization 3×10^6 and 6×10^6 cells for each time point were assayed for ribonucleotide reductase activity (10).

which was slightly smaller than in 3T6 cells. Earlier experiments with different cell lines (13) have suggested that large dCTP pools, and high dCTP/dATP ratios, are characteristic for cells in late G1 or early S phase. While this is true for 3T6 cells, the resistant cells clearly do not abide by this rule.

Subunit Composition of Reductase from Resistant Cells. The increase in reductase activity in extracts from resistant cells can have several alternative explanations: (*i*) overproduction of the complete enzyme; (*ii*) overproduction of one of the two subunits; or (*iii*) activation of the enzyme or one of its subunits.

The amount of M1 subunit present in extracts was determined in two independent ways. In the first method, extracts from 3T6 or resistant cells were electrophoresed directly on $NaDodSO_4$ gels, and the bands were transferred to diazobenzyloxymethyl-paper and allowed to react first with rabbit antibody to thymus protein M1 and then with ^{125}I-labeled staphylococcal protein A. The radioactivity recovered in the band at the position of M1 is then a measure of the amount of this subunit present in the original extracts, because the antibody against thymus M1 completely neutralized the reductase activity also in the extract from resistant cells. As shown in the inset of Fig. 4, there was no apparent difference between extracts from 3T6 and resistant cells.

In the second approach the proteins of the extracts were labeled by growing cells in media containing either ^3H- or ^{14}C-labeled leucine. Each extract was first passed through a column of dATP-Sepharose and the adsorbed M1 subunit was then eluted specifically with 1 mM dATP. In both cases the material eluted specifically from dATP-Sepharose contained 0.5% of the total radioactivity present in the extract. The eluates were then electrophoresed on $NaDodSO_4$ gels and the radioactivity present in the position of M1 was a measure of the amount of this subunit in the original extract. In Fig. 4 a distinct radioactive peak is found at the position of the M1 subunit. In both cases this peak corresponds to 0.07% of the original total radioactivity. It should be pointed out that dATP similarly inhibited the activity of the reductase from each extract, assuring a similar affinity of protein M1 for dATP-Sepharose (data not shown).

A corresponding direct approach could not be made to determine the amount of M2 subunit present in the extracts. We discovered, however, that the mouse M2 subunit was complemented by the thymus M1 subunit, and this made possible a comparison of the M2 activities in the two extracts (Fig. 5). In both cases addition of an excess of M1 from thymus greatly increased the reduction of CDP. At saturation, the activity of protein M2 was 27 times higher in the extract from the resistant line.

Attempts to demonstrate that this substantial increase in subunit activity was due to overproduction of a specific protein were not successful. However, by analogy with the tyrosine free radical in the B2 subunit of the *E. coli* enzyme, we considered the possibility that a free radical is present in M2 also. Previously we had used the characteristic EPR signal of the tyrosine radical to demonstrate the presence of the radical in a strain of *E. coli*

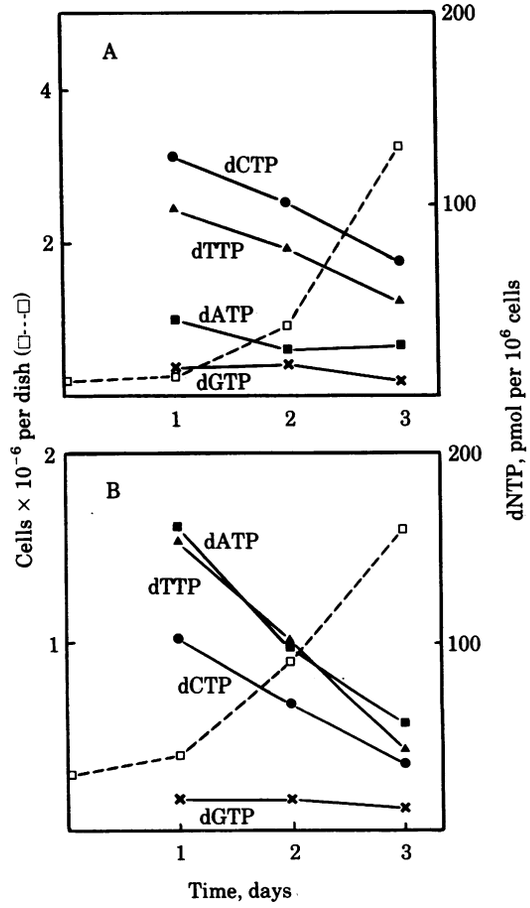

FIG. 3. dNTP pools during growth of 3T6 (*A*) and resistant 3T6-HU-11 (*B*) cells. The two cell lines were grown on 5-cm dishes and extracted at the indicated times with 60% (vol/vol) methanol for the determination of the four dNTP pools (11, 12).

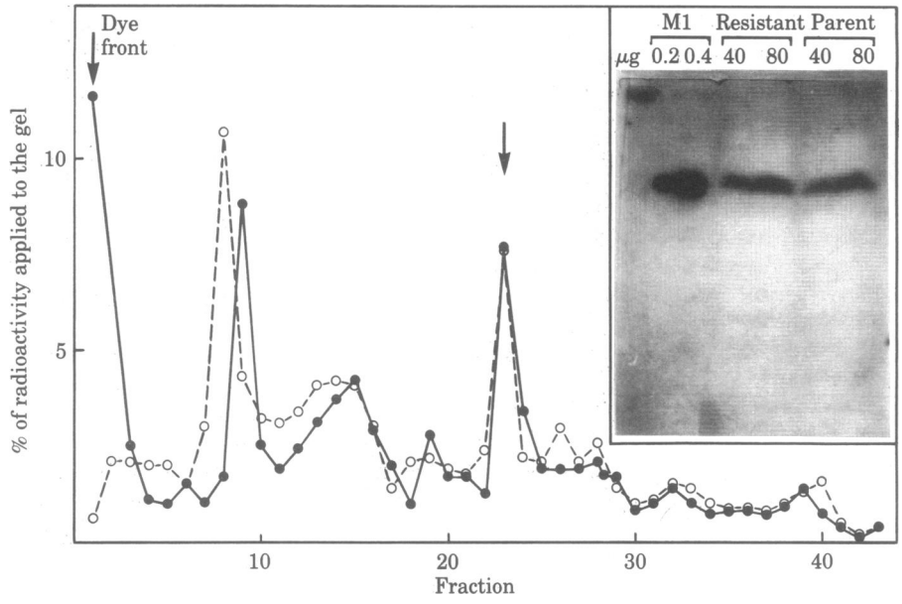

FIG. 4. Amount of protein M1 in extracts from parent and resistant cells. Pulse-labeled soluble proteins (0.8 mg containing 3.6×10^6 cpm) from resistant (●) and parent (○) cells were chromatographed on two columns of dATP-Sepharose (0.4 × 1.6 cm) as described (2). The adsorbed M1 protein was eluted specifically with 1.0 ml of 1 mM dATP. After addition of 100 μg of bovine serum albumin carrier, the proteins in the eluates (17,300 cpm) were precipitated with trichloroacetic acid, dissolved in $NaDodSO_4$ sample buffer, and electrophoresed on polyacrylamide/$NaDodSO_4$ tube gels (0.6 × 9 cm) as described in ref. 2 for slab gels. The gels were sliced in a Gilson gel fractionator, extracted overnight at 37°C in 0.5 ml of 50 mM Tris·HCl, pH 7.6/0.2% $NaDodSO_4$, and analyzed for radioactivity. The arrow shows the position of protein M1 (polypeptide molecular weight 84,000). (*Inset*) Identical amounts of protein (150 μg) in extracts from parent or resistant cells were precipitated with trichloroacetic acid and dissolved in $NaDodSO_4$ sample buffer as described (2). Pure protein M1 from thymus (0.2 and 0.4 μg) together with 40 and 80 μg of protein from each cell line was electrophoresed directly on a $NaDodSO_4$/polyacrylamide slab gel containing ethylene diacrylate as a crosslinker (14). After electrophoresis the protein bands were transferred by blotting to diazobenzyloxymethyl-paper (14) and the paper was incubated overnight at 37°C with 0.7 mg of gamma globulin from a rabbit immunized with thymus protein M1, followed by ^{125}I-labeled protein A (4.1 μCi, specific activity 6.5 μCi/μg). Autoradiography was on Kodak X-Omat R film in combination with a Kodak X-Omatic regular screen at −70°C for 24 hr.

overproducing the reductase by measuring packed cells directly (8). We therefore centrifuged cells from the resistant and parent lines of the mouse fibroblasts in EPR tubes and recorded their EPR spectra. The results in Fig. 6 clearly demonstrate that an EPR spectrum of a free radical can be observed in the resistant line, but not in the corresponding parent line (at least a 20-fold excess in the resistant cells). Furthermore, this EPR spectrum is closely similar to, although not identical with, the EPR spectrum of the tyrosine radical of protein B2. Thus there seems to

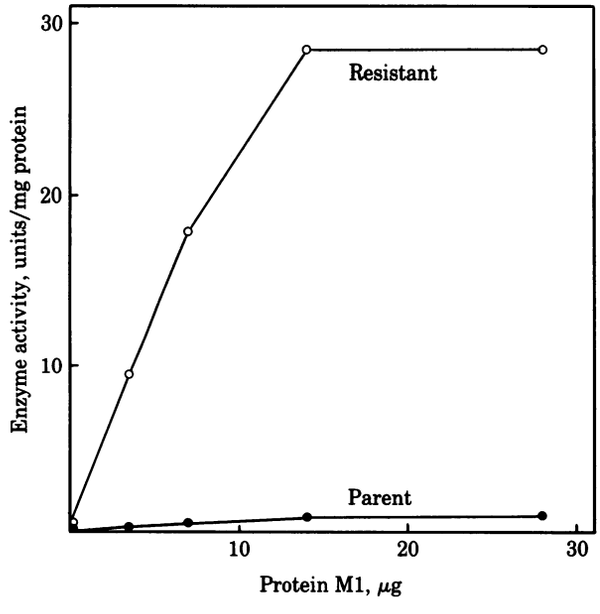

FIG. 5. Determination of M2 activity in extracts from parent and resistant cells. Ribonucleotide reductase activity was measured in two series of incubation mixtures containing a constant amount of cell extract (7 μg and 32 μg of protein from resistant and parent cells, respectively) and increasing amounts of pure thymus protein M1 as indicated.

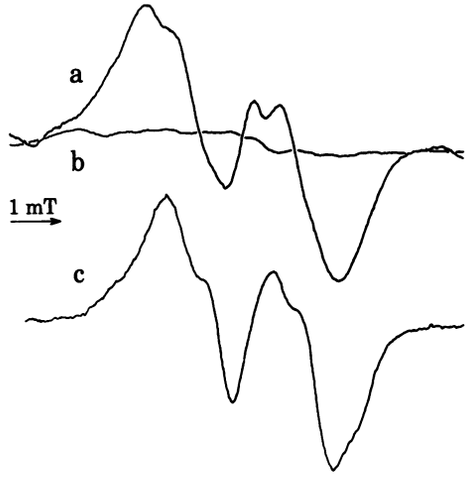

FIG. 6. EPR spectra at 77 K of packed samples of resistant (spectrum a) and parent (spectrum b) line mouse fibroblast cells, and *E. coli* cells overproducing ribonucleotide reductase (spectrum c) (8). EPR conditions for spectrum a: modulation amplitude 0.19 mT, microwave power 65 mW, scanning rate 2 mT/min, detector time constant 1 s; for spectrum b: the same as for a except scanning rate 1 mT/min and detector time constant 3 s; for spectrum c: the same as for b except modulation amplitude 0.15 mT and microwave power 0.3 mW.

be little doubt that the M2 subunit contains a tyrosine free radical, similar to that of protein B2. After incubation of the resistant cells with 50 mM hydroxyurea for 15 min the spectrum disappeared completely (data not shown).

From the spin concentration of the free radical we can calculate that protein M2 amounts to 0.17% of the total extractable protein. This calculation assumes the presence of one radical per polypeptide chain of molecular weight 55,000 and represents only a rough estimate.

DISCUSSION

A key involvement of ribonucleotide reductase in the development of resistance against hydroxyurea in mammalian cells was already suggested from the experiments of Lewis and Wright (15). Resistant Chinese hamster ovary cells were found either to acquire an enzyme with a decreased sensitivity towards the drug or to overproduce enzyme activity (6). The mechanism for the overproduction was not clarified. Other similar cases of drug resistance had, however, been shown to be due to gene duplications resulting in overproduction of specific messenger RNAs and corresponding target enzymes (4, 5).

Our experiments with 3T6 cells and in particular the data obtained for the "revertant" line (HU-11R) shown in Fig. 1 are further supportive evidence for ribonucleotide reductase being the target for the inhibition of cell growth and DNA synthesis by hydroxyurea. We found that the cells acquired resistance against the drug by an increase in enzyme activity. However, our data strongly suggest that this increase was not caused by overproduction of the complete enzyme. This conclusion may have implications for the normal regulation of ribonucleotide reductase during cell growth.

The work of Thelander *et al.* (3) firmly established that the thymus enzyme consists of two nonidentical subunits, which were called M1 and M2. Only the M1 subunit has so far been obtained in pure form and characterized as a protein. This subunit is not overproduced in our resistant cell line, and from radioactivity data we estimate that 0.07% of the total protein in extracts from either line corresponds to M1. Because of possible losses during extraction and chromatography we consider this value to be a minimum value.

The amount of protein M2 could not be determined by a direct method. However, the EPR data shown in Fig. 6 establish that the resistant cells contained more than a 20-fold increase of a free radical that in the case of the *E. coli* reductase is known to be a tyrosine radical, specific for the B2 subunit (16) and therefore in all probability also specific for the mammalian M2 protein. The parallel increase in M2 activity in extracts from resistant cells supports such a conclusion. Furthermore, because hydroxyurea is known to inhibit the bacterial reductase by interaction with its tyrosine radical (8), the observed overproduction appears to provide the explanation for the development of resistance.

The increase in free radical can arise from either of two alternative mechanisms: (*i*) overproduction of the complete M2 protein, or (*ii*) overproduction of the radical within the M2 protein. In the latter case the parent and resistant lines both would contain similar amounts of M2 protein and differ only with respect to the amount of free radical. Such a correlation between amount of free radical and enzyme activity was observed earlier for the B2 subunit of the *E. coli* reductase (17).

In the resistant line, at least 0.07% of the extractable protein corresponded to protein M1 and roughly 0.17% to protein M2, suggesting some molar excess of M2 over M1. However, the uncertainties involved in our calculations do not exclude the possibility that both subunits occur in stoichiometric amounts. The parent line contains less than 5% of the free radical content of the resistant line and therefore appears to have a large excess of protein M1 over active M2.

This argument opens up interesting possibilities for normal control mechanisms of ribonucleotide reductase. This enzyme shows large variations in its activity in different cells (18) and during the growth cycle (19). Such variations were thought to be due to rapid synthesis and breakdown of the complete enzyme. Our results now suggest that they might be caused either by rapid variations in the amount of tyrosine radical within a "dormant" M2 subunit or by the specific synthesis and breakdown of only this subunit.

Our final point concerns the measurements of the deoxynucleoside triphosphate pools depicted in Fig. 3. The increased enzyme activity in the resistant lines resulted in a 3- to 4-fold increase of the dATP pool but only in small increases of dTTP and dGTP, while the dCTP pool actually showed a slight decrease. It is possible to relate this result to the known allosteric properties of the enzyme, which depend on the binding of effectors to the M1 subunit (3, 9). It should also be kept in mind that the amount of M1 is not increased in the resistant line. Binding of dATP to M1 is known to turn off the activity of the reductase. In the resistant cell the potential for an increased reduction of ribonucleotides derived from the large increase of tyrosine radical is therefore tempered by binding of the excess of dATP to the M1 subunit.

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