The Effect of 3H-Thymidine on the Proliferation of in vitro Cultured Mammalian Cells

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Abstract. The effect of 3H-thymidine on the proliferation of Chinese hamster cells (clone V79) was studied. Following 3H-thymidine application the proliferation of cells (studied on the basis of plating efficiency) was found to be diminished, the drop being dependent on radioactivity (2—20 kBq/ml cultivation medium), the time of application (2—20 h) and specific activity of 3H-thymidine added. Exogenous macromolecular DNA was able to repair, to an important degree the radiotoxic effect of 3H-thymidine on V79 cells by a mechanism other than the mere reduction of specific activity of 3H-thymidine.

Key words: Chinese hamster cells (clone V79) — DNA — 3H-thymidine — Plating efficiency — Autoradiography

Introduction

The radioisotope technique is very extensively used in research and clinical practice to study the incorporation of various substances labelled with an isotope into cells and tissues. One of the frequently used isotopes is tritium, to study e. g. the synthesis of DNA, to establish generation phases of cell populations, etc. For this purpose 3H is incorporated into the DNA precursor thymidine. The interpretations based on studies of the 3H-thymidine incorporation into cell DNA have been based on the assumption that the decay of the incorporated 3H would not affect the cell functions. It has, however, been found that the decay of 3H incorporated into the cell DNA can cause breaks in the DNA (Kupersztoch-Portnoy and Helinski 1974; Sundell-Bergman and Johanson 1980, 1982; Nes 1981), chromosome aberrations (Chan et al. 1976; Burki et al. 1975) or exchange of sister chromatids (Gibson and Prescott 1972), and may even lead to neoplastic transformation of cells (Lin et al. 1982). Beck (1982) has reported that the incorporation of 3H-thymidine into the DNA of mammalian cells may also result in a prolongation of the S, G_2 and M phases, respectively, the total prolongation being up to 20 h.

Our experiments were therefore, concentrated on the study of the effect of 3H-thymidine on the proliferation of Chinese hamster cells (clone V79) in dependence on low radioactivities of 3H-thymidine, i. e. radioactivities such as

those used in routine biochemical tests. Since it is known that exogenous DNA has repairing capacity in cells damaged by X-rays (Minářová and Keprtová 1978), we also tried to establish whether exogenous DNA would also repair damage due to the incorporation of 3H-thymidine into the mammalian cell DNA.

Materials and Methods

Cultivation of cells: An aneuploid line of Chinese hamster cells (clone V79) were used. They were grown on a monolayer in minimal Eagle's medium (MEM) plus 10 % bovine serum and antibiotics.

Plating efficiency: One thousand cells were plated on 10 ml MEM with 20 % bovine serum, in plastic Petri dishes (⌀ 60 mm). Four hours after plating 3H-thymidine (Thymidine-6-3H 980 GBq/mM, ÚVVVR Praha), at a concentration of 2—20 kBq/ml medium, or 3H-thymidine and exogenous DNA (10 μg/ml medium) were added to the cells. In a further series of experiments, 3H-thymidine in a single concentration (4 kBq/ml) was added to cells together with various concentrations of non-labelled thymidine (Calbiochem). Colonies of cells with no 3H-thymidine added served as controls.

After various periods of incubation (2—20 h) the medium was replaced with a fresh one containing no 3H-thymidine, nonlabelled thymidine or exogenous DNA. The cells were cultured in this environment for further 6 days. The medium was then removed, the colonies were dried at room temperature and stained with 10 % Giemsa-Romanowski solution. The values given in the graphs represent the means of 16 samples (each experiment with four samples repeated four times).

Autoradiography: 4×10^5 cells were plated on a cover slip in a Petri dish (⌀ 50 mm) with 5 ml MEM plus 10 % bovine serum. After incubation with 3H-thymidine, non-labelled thymidine or exogenous DNA cells were washed with Hanks solution and fixed with an ethanol — concentrated acetic acid (3 : 1) solution. The acidosoluble material was removed with 1 % chloric acid at 4 °C and a solution of non-labelled thymidine (2.5×10^{-2} g/l). The cover slips were coated with an emulsion (Ilford L4), exposed for 20 h and developed using Kodak D 19 developer. The cells were stained with methyl-green-pyronine. The mean numbers of grains per cell for 100 cells and the percentage of radioactive cells for 1000 cells were calculated.

Isolation of DNA: DNA was isolated from V79 cells or mouse fibroblast cells of the L strain by the method according to Marmur (1961) modified for mammalian cells (Keprtová 1973). The isolated DNA had a mean molecular weight of 10^7 Daltons. Both macromolecular DNA and DNA degraded to its acidosoluble products with DNase (Calbiochem) were used. The application procedure of exogenous DNA is given for individual experiments.

Results

The effect of 3H-thymidine on cells was studied in dependence on: a) the time of application, b) radioactivity (kBq/ml medium) and c) specific activity.

The effect of 3H-thymidine radioactivity and application time. 3H-thymidine was applied to cells for periods of 2; 4; 10; or 20 h. Activities of 2; 4; 8; 16 or 20 kBq/ml medium were used. The controls were cells with no 3H-thymidine added. As can be seen from Fig. 1, the effect of 3H-thymidine depends on both its

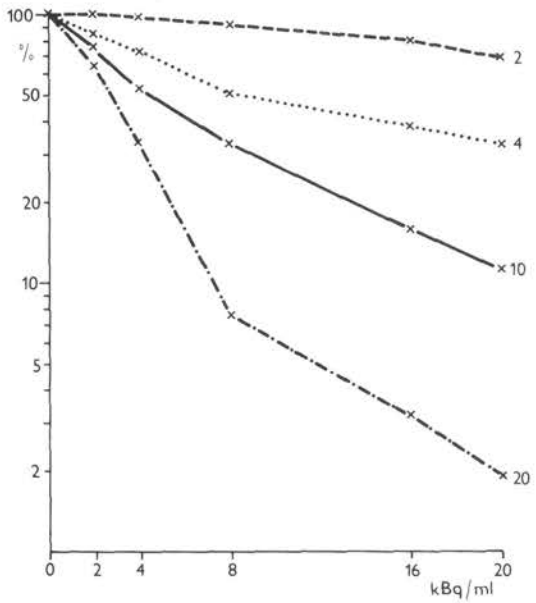

Fig. 1. The effect of 3H-thymidine concentration and application time on growth of V79 cells. Four hours after plating the cells, 3H-thymidine was applied in concentrations given on the abscissa for periods of 2 (– – –); 4 (· · · ·); 10 (——); or 20 (–·–·) hours. Ordinate: relative values; 100 % is the number of colonies growing in cells without the addition of 3H-thymidine (515). After incubation with 3H-thymidine, the medium was replaced by fresh medium without 3H-thymidine, and the cells were cultured for further 6 days. The number of colonies was statistically significantly decreased during incubation for 2 h after a dose of 20 kBq/ml, for 4 h after 4 kBq/ml, and during incubation for 10 and 20 h after all above mentioned concentrations of 3H-thymidine.

radioactivity and the time of application. At the shortest time interval used (2 h) a major decrease in colony formation could be observed at the highest radioactivity of 3H-thymidine (20 kBq/ml) only, (32 %). Application of 3H-thymidine to cells for periods longer than the generation time (20 h), resulted in a reduction of the number of colonies by 36 % even with the lowest 3H-thymidine radioactivity (2 kBq/ml); with 8 kBq/ml, only 8 % of the number of colonies was formed as compared to the control samples with no 3H-thymidine added.

The effect of exogenous DNA on cells following previous 3H-thymidine application. Both isologous and heterologous (the latter both macromolecular and degraded), exogenous DNA (10 μg/ml) was used. For comparison, non-labelled thymidine was applied to a further series of samples at a concentration corresponding to the amount of thymidine in 10 μg of exogenous DNA (1.8 μg/ml). The samples to which only DNA or non-labelled thymidine had been added were used

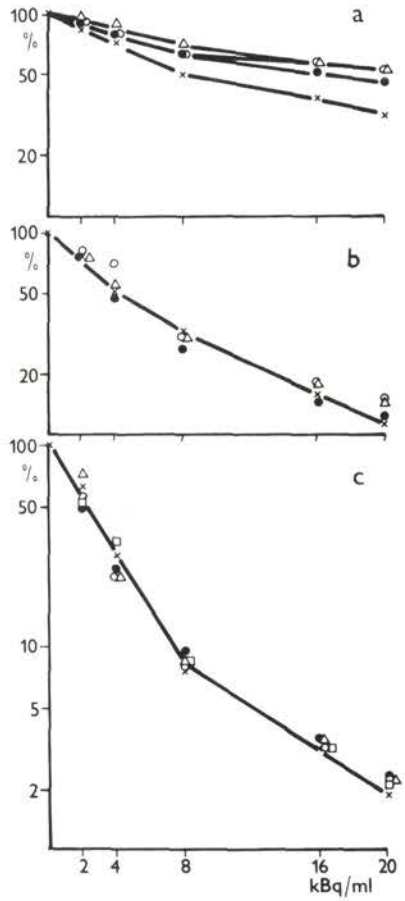

Fig. 2. The effect of exogenous DNA and nonlabelled thymidine on the growth of V79 cells after the application of 3H-thymidine. 3H-thymidine was applied for 4 (*a*); 10 (*b*); or 20 (*c*) hours. The medium was then replaced by a fresh one containing either no (×) or 10 μg/ml exogenous DNA, either heterologous (○) of isologous (□) or acidosoluble products of DNA degradation (△), or nonlabelled thymidine 1.8 μg/ml (●). Ordinate: relative values; 100 % is the number of colonies growing in the absence of 3H-thymidine (515). Isologous DNA was applied only in case 2c. Abscissa: 3H-thymidine concentration. Addition of exogenous DNA or of nonlabelled thymidine will have no statistically significant influence on the number of colonies even during a 4 h incubation.

as controls. The addition of these latter substances had no effect on the number of colonies; colony numbers in these samples were similar to those in samples to which none of the substances mentioned were added (an average of 515 colonies). In studying the effect of exogenous DNA on colony formation in cells with 3H-thymidine added we found that both the amount of 3H-thymidine radioactivity

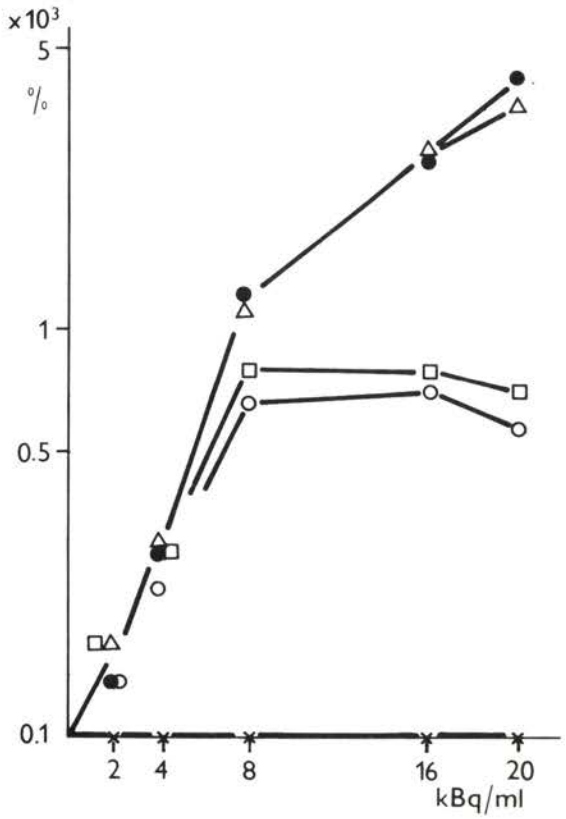

Fig. 3. The growth of V79 cells after the application of exogenous DNA or nonlabelled thymidine added to the cells immediately after the application of 3h-thymidine. Within 10 min of the application of 3H-thymidine either nonlabelled thymidine (1.8 μg/ml) (●) or 10 μg/ml heterologous (○), isologous (□) or degraded (△) exogenous DNA was added to the cells. After 20 h incubation with these substances the medium was replaced by a fresh one and the number of colonies were counted after 6 days of incubation. Controls (shown on abscissa (×)) are the numbers of colonies growing in samples with 3H-thymidine alone. Abscissa: 3H-thymidine concentration. Ordinate: shows percent by which the numbers of colonies after application of the various substances are increased as compared with controls (= 100 %). Nonlabelled thymidine and degraded DNA will statistically significantly increase the number of colonies already after the application of 2 kBq 3H-thymidine/ml of culture medium. Macromolecular DNA of both isologous and heterologous origin will statistically significantly increase the number of colonies only after the application of 4 kBq 3H-thymidine/ml of culture medium.

and the interval between the application of 3H-thymidine and DNA is important. If exogenous DNA, whether macromolecular or degraded, or nonlabelled thymidine were applied only after 10 or 20 h following the addition of 3H-thymidine, these substances had no effect on plating efficiency (Fig. *2B, C*). If

exogenous DNA or nonlabelled thymidine were applied 4h after the application of 3H-thymidine, the number of colonies slightly increased following the application of these substances, at any of the 3H-thymidine radioactivities used (Fig. 2*A*).

If, after the application of 3H-thymidine to cells, exogenous DNA (isologous, heterologous or degraded) or nonlabelled thymidine were added within 10 min, the number of colonies increased considerably the more, the higher was the radioactivity of 3H-thymidine. Fig. 3 shows the increase in the number of colonies after the application of the above substances as compared with samples with only 3H-thymidine added. As can be seen, the greatest numbers of colonies could be observed after the application of nonlabelled thymidine or degraded DNA; this means that the lower the specific activity of 3H-thymidine the weaker its radiotoxic effect on cells. It also follows from these results that exogenous DNA in the macromolecular form has also a considerable positive effect on plating efficiency, no imporant difference appearing between the effects of isologous and heterologous DNA.

To find out to what extent the positive effect of DNA may be caused by its degradation to nucleosides and consequent lowering of the specific activity of 3H-thymidine, we further studied the plating of V79 cells in dependence on the specific activity of 3H-thymidine, and compared the results with the effect of exogenous DNA.

The effect of the specific activity of 3H-thymidine on cell growth. The same 3H-thymidine radioactivity (4 kBq/ml medium) was used throughout this series of experiments, with its specific activity being altered by the addition of various concentrations of nonlabelled thymidine to the cultivation medium. The amount of thymidine contained in 4 kBq 3H-thymidine, i. e. 1 ng, was taken as the basis. Nonlabelled thymidine was then added to individual samples to obtain reductions of 3H-thymidine specific activity 0.1×; 0.2×; 1×; 2×; 10×; 20×; 100×; 200×; 1000×; and 2000×. Another series of samples were run simultaneously with exogenous macromolecular DNA (10 μg/ml) added in addition to the above concentrations of nonlabelled thymidine. The cells were cultured in this environment for 20 h, since at this time, the radiotoxic effect of 3H-thymidine is the strongest. As can be seen in Fig. 4, the reduction in the specific activity of 3H-thymidine had no effect on the number of colonies up to a factor of ten. There was a considerable increase in the number of colonies only at 100-fold reduction of the specific activity of 3H-thymidine.

In samples with exogenous DNA added the pattern was quite different. High number of colonies (over 500) were obtained after the application of exogenous DNA alone. The addition of nonlabelled thymidine brought about only a slight additional increase, i. e. an increase in the number of colonies as compared with the addition of exogenous DNA alone, at the two highest concentrations of nonlabel-

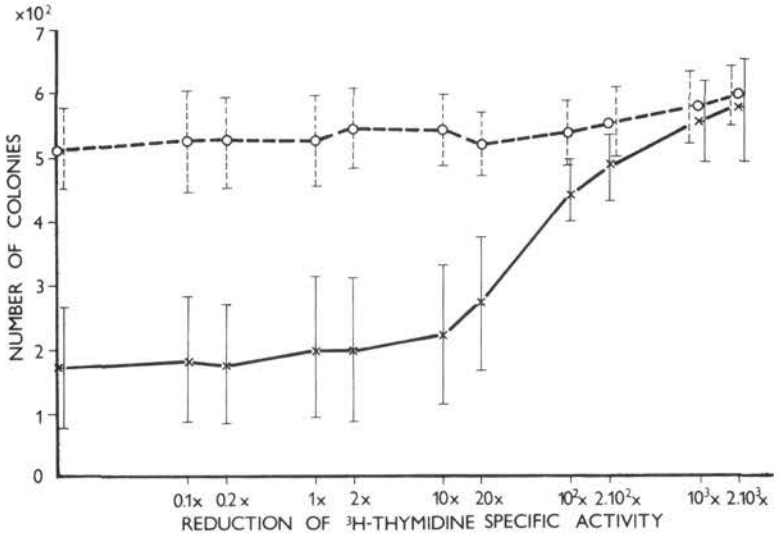

Fig. 4. The effect of the specific activity of 3H-thymidine on the growth of V79 cells. Four kBq/ml of 3H-thymidine was applied to each sample. To one half of the samples, various concentrations of nonlabelled thymidine were added, and to the other half nonlabelled thymidine plus exogenous macromolecular DNA (10 μg/ml) were added. Abscissa: shows reduction of the specific activity of 3H-thymidine based on the amount of thymidine found in 4 kBq 3H-thymidine (= 1). The ordinate shows the number of colonies. Full line indicates samples with nonlabelled thymidine, broken line represents samples with nonlabelled thymidine plus exogenous DNA. In control samples (without 3H-thymidine), 639 colonies were counted. A statistically significant increase in the number of colonies with respect to samples with 3H-thymidine alone will occur after the application of exogenous DNA and after a 100 fold reduction of 3H-thymidine specific activity.

led thymidine plus exogenous DNA only (i. e. in dilutions 1000 × and 2000 ×).

Exogenous DNA alone increased the numbers of colonies over those in samples to which 3H-thymidine alone was added, by a factor of three. A similar increase in the number of colonies occurred after the addition of an amount of nonlabelled thymidine corresponding to that in 2.5 μg exogenous DNA. If the positive effect of exogenous DNA on cells were due only to a decrease in the specific activity of 3H-thymidine, then at least 25 % of the exogenous DNA would have to be very rapidly degraded into nucleosides. However such rapid degradation of exogenous DNA does not take place in cultivation medium (Keprtová 1973).

The establishment of degradation of exogenous DNA after its entering the cell. Using the autoradiographic technique we tried to find out whether or not exogenous DNA is degraded only after its entering the cell. If this were true,

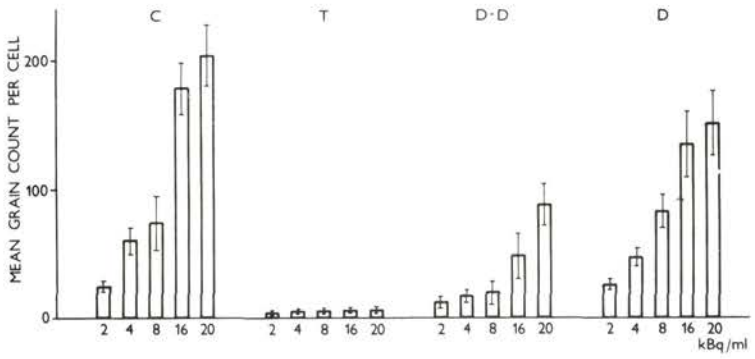

Fig. 5. Autoradiographic assessment of the amount of 3H-thymidine incorporated into cells. 3H-thymidine was applied in quantities given for 20 h (C). In addition to 3H-thymidine macromolecular (D) or degraded (D-D) DNA (10 μg/ml) or nonlabelled thymidine — 1.8 μg/ml (T) are immediately added to the cells. The abscissa gives the concentrations of 3H-thymidine, the ordinate gives the mean grain counts per cell; horizontal bars indicate standard deviation. A statistically significant decrease in the mean grain count per cell was found in all cases after the application of nonlabelled thymidine and degraded DNA; the same was found after the application of macromolecular DNA in samples with 16 and 20 kBq 3H-thymidine/ml of culture medium.

exogenous DNA would decrease the specific activity of 3H-thymidine only after entering the cell, but prior to its incorporation into the cell DNA. In this series of experiments 3H-thymidine with radioactivities of 2; 4; 8; 16; and 20 kBq/ml medium was added to V79 cells; also, exogenous DNA either in macromolecular or degraded form (10 μg/ml) and nonlabelled thymidine (1.8 μg/ml, corresponding to the amount of thymidine in 10 μg DNA) were added to a portion of the samples. The cells were cultured in this environment for 20 h. The amount of radioactive cells was above 90 % in all cases, with the exception of the sample with nonlabelled thymidine and the lowest 3H-thymidine radioactivity (2 kBq/ml), 80.5 % of radioactive cells in the population in the latter case. In all samples with nonlabelled thymidine added to 3H-thymidine, the mean grain counts per cell were only slightly higher than the background (4—5 grains), (Fig. 5). If degraded DNA was added to cells after the application of 3H-thymidine, the specific activity of 3H-thymidine was considerably decreased. However if nonlabelled exogenous macromolecular DNA was added together with 3H-thymidine, the mean grain counts per cell decreased by one quarter only after the application of the two highest radioactivities of 3H-thymidine (16 and 20 kBq/ml). At the same time, however, in both cases the grain counts per cell were 29 times higher than in the samples with nonlabelled thymidine.

We further used the autoradiographic technique to try to establish a dependence of the incorporation of 3H-thymidine into cells on its specific activity. We therefore studied the number of radioactive cells and the mean grain count per cell

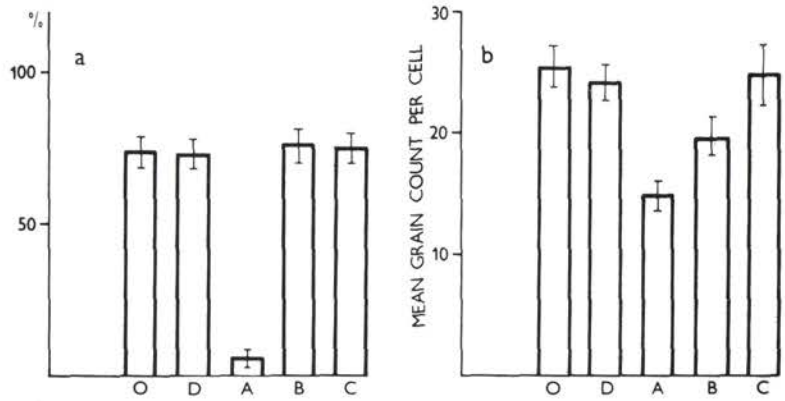

Fig. 6. The amount of 3H-thymidine incorparated into cells in dependence on its specific activity. 3H-thymidine was added to cells for 4 h at a concentration of 4 kBq/ml. The autoradiographic technique was used to establish the number of radioactive cells (*a*) and the mean grain counts per cell(*b*). O — 3H-thymidine alone, D — 3H-thymidine added together with 10 μg/ml of exogenous macromolecular DNA; the remaining samples had nonlabelled thymidine added together with 3H-thymidine, (9×10^{-1} (A), 9×10^{-2} (B) and 9×10^{-3} (C) μg/ml). The ordinates show the percentages of radioactive cells (*a*) and the mean grain counts per cell (*b*). Horizontal bars indicate standard deviation. A statistically significant decrease in the percent of labelled cells was found only in A samples; the mean grain count per cell in samples A and B was statistically significantly decreased as against control samples (0).

after the incorporation of 3H-thymidine alone, 3H-thymidine together with 10 μg/ml of exogenous macromolecular DNA, or 3H-thymidine, with nonlabelled thymidine amounts corresponding to 50 % (A), 5 % (B) or 0.5 % (C) of the amount of thymidine found in 10 μg exogenous DNA were used. In all samples 3H-thymidine with a radioactivity of 4 kBq/ml medium was used. The cells were incubated with above substances for 4 h. It can be seen from Fig. 6 that the number of cells capable of incorporating 3H-thymidine was decreased only after the application of the highest concentration of nonlabelled thymidine. The amount of 3H-thymidine found in the cells (shown as the mean grain count per cell) was affected by the addition of nonlabelled thymidine corresponding to 5 % (B) or 50 % (A) of the thymidine content in the exogenous DNA, respectively. In samples with 3H-thymidine added together with exogenous DNA, the percentage of radioactive cells and the grain count per cell remained approximately the same as in the controls (with only 3H-thymidine added), the slight decrease in the mean grain count per cell after the application of exogenous DNA (7 % — statistically insignificant)was much smaller than after the application of nonlabelled thymidine at a concentration corresponding to 5 % DNA degraded to nucleosides (here, the decrease was 28 % as compared with controls). This means that less than 5 % of

exogenous DNA degraded during the incubation, both in the cultivation medium and after the incorporation of the exogenous DNA into the cell.

Discussion

Unlike other types of ionizing radiation, such as X-rays, which irradiate the whole cell, tritium when applied to mammalian cells in the form of 3H-thymidine, is incorporated into an important biomacromolecule, DNA. Here it induces various changes which may lead to considerable variations in the proliferation kinetics and even to somatic mutations which bring about a neoplastic transformation (Lin et al. 1982). These authors presume that the neoplastic transformation of embryonic Chinese hamster cells is due to the position of tritium in the DNA molecule, which may induce changes leading either to derepresion of a strictly regulated gene, or to expression of a normally unexpressed gene. This mutation need not, however, occur in another cell line, since not all cell lines are equally sensitive to the β particles emitted by tritium (Beck 1981; Bedford et al. 1975; Wiezsaecker et al. 1981).

Since changes in the proliferation kinetics of mammalian cells may lead to an erroneous interpretation of results obtained using the incorporation of 3H-thymidine into cellular DNA, we studied the proliferation of mammalian cells cultured in vitro. We paid great attention to the effect of low radioactivities of 3H-thymidine, i. e. those routinely used in radiobiochemical measurements. As already mentioned in the Results section, even 3H-thymidine radioactivity as low as 4 kBq/ml cultivation medium (approximately 0.1 μCi/ml) decrease the number of viable cells to one third if 3H-thymidine is applied for period longer than the generation time of the cells (about 20 h). From our observation of the effect of the specific activity of 3H-thymidine on the proliferation of V79 cells it is clear that the use of the lowest 3H-thymidine specific activity possible is advantageous.

Exogenous polymeric DNA repairs damage arising from the incorporation into cells of 3H-thymidine; this repair capacity increases with the increasing radioactivity of 3H-thymidine (from 2 to 20 kBq/ml). Principally, no difference was found between the effects of isologous and heterologous DNA. It is, however, important that DNA is applied within a shortest interval possible after 3H-thymidine. This would indicate that in the medium exogenous DNA degrades to nucleosides, which in turn change the specific activity of 3H-thymidine, thus reducing its radiotoxic effect to the cells. However, as can be seen from Fig. 3, the radiotoxic effect of 3H-thymidine is decreased to a much greater extent after the application of degraded DNA than after the application of polymeric DNA. From this fact it follows that far from all of the exogenous polymeric DNA degrades, after its addition to the cultivation medium, to nucleosides that could reduce the specific activity of 3H-thymidine. Moreover, is known that, after 4 h incubation of

exogenous polymeric DNA in the cultivation medium (when its repair effect on cells is already small — see Fig. 2*A*), only less than 10 % of exogenous DNA degrades in the cultivation medium (Keprtová 1973).

However, exogenous DNA may intensively degrade only after its incorporation into the cell. We therefore studied the dependence of the proliferation of mammalian cells on the specific activity of 3H-thymidine and compared it with the effect of exogenous DNA. However our results showed (Fig. 4), that more than 25 % of the exogenous DNA would have to split up to nucleosides, since this is the amount of nonlabelled thymidine which has the same effect on cell proliferation (after the application of 3H-thymidine) as exogenous DNA.

Using autoradiographic technique we also could show that in the course of 20 h incubation exogenous polymeric DNA decreases the incorporation of 3H-thymidine in the cells by an 11 % in average. If 3H-thymidine is applied for a short period (4 kBq/ml, 4 h), the exogenous macromolecular DNA has almost no effect on its incorporation into the cells. It follows from a comparison with the effect of nonlabelled thymidine that, oven after incorporation into the cell, less than 5 %, of the exogenous DNA is degraded, which agrees with our previous findings (Keprtová and Minářová 1982). This means that, oven after entering the cell, the degree of degradation of exogenous DNA is insufficient to explain the occurrence of the entire repair effect on cells damaged by the incorporation of 3H-thymidine into cell DNA. Based on this it can be supposed that exogenous polymeric DNA repair cells damaged due to 3H-thymidine incorporation into DNA by decreasing its specific activity, and by another mechanism which may be similar to that observed in cells damaged by X-rays (Minářová and Keprtová 1978).

Painter and Young (1974) suggest that the repair of damage caused by tritium incorporated into cell DNA has a similar mechanism to that occurring in cells damaged by external radiation. Certain differences in the effects of X-rays and tritium have, however, been found. Panter (1981) found, for example, that unlike the X-ray damage, that caused by the incorporation of tritium is not dependent on temperature and that the decay of tritium kills cells at − 196 °C to a much greater extent than X-rays. He further states that 3H in the DNA molecule inactivates the cells more than the equivalent dose of X-rays in rads. It is therefore essential to bear in mind that there exists a certain risk in using 3H-thymidine for labelling mammalian cells due to the degree of erroneous interpretation of the results obtained, since even a very low 3H-thymidine radioactivity brings about changes in the proliferation of mammalian cells.

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