to Wilson et al. [15], the number of stromal precursor cells in the contralateral femur of curetted mice on the 11th day was increased by four times. These results agree with those of autoradiographic analysis of bone marrow [10], which showed that partial depopulation of the medullary cavity leads to stimulation of the proliferative activity of stromal cells in undamaged areas.

The mechanism and role of this systemic response of the stromal tissue have not yet been explained and are interesting for they indicate the existence of hitherto unknown interrelationships between different parts of the microenvironment of hematopoietic tissue mediated through humoral or nervous connections.

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FORMATION OF DNA-PROTEIN CROSS-LINKAGES AND THEIR POSSIBLE ELIMINATION BY MUSTINE IN CULTURES OF NORMAL HUMAN FIBROBLASTS

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To determine DNA-protein cross-linkages induced by mustine in cultures of human skin fibroblasts a radiometric method and fractionation of the cell lysate on hydroxyapatite were used. The formation of DNA-protein cross-linkages and their elimination during long-term culture of the cells after treatment with the mutagen were demonstrated.

KEY WORDS: mustine; DNA-protein; repair.

The formation of DNA-protein cross-linkages in the chromatin of eukaryotes is evidently a general type of injury which may arise through exposure to various physical and chemical mutagens: ionizing radiation [5], UV radiation [13], and alkylating compounds [5, 7-12, 14, 15]. According to our own observations, these injuries cause disturbances of the template properties of chromatin [4]. Methods used currently to detect DNA-protein cross-linkages in cells are indirect and do not provide an unequivocal answer to the question of the quantitative parameters which characterize this defect. In the present investigation, in which a radiometric method developed

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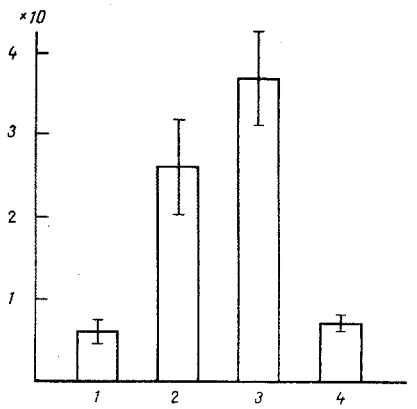

Fig. 1. Changes in ratio between levels of amino acid (^{14}C) and thymidine (^{3}H) radioactive labels in DNA fraction eluted from HAP by solvent consisting of 0.4 M phosphate buffer + 6 M urea + 2.5 M NaCl. 1) Control (culture of fibroblasts not treated with mustine) – mean of eight repetitions; 2) immediately after removal of mustine (mean of eight repetitions); 3) 5 h after removal of mustine (mean of five repititions); 4) 72 h after removal of mustine (mean of four repititions). Ratio between amino acid and thymidine radioactive labels determined by the formula

$$\frac{(^{14}C) - \text{background } (^{14}C)}{(^{3}H) - \text{background } (^{3}H) - K(^{14}C)}$$

where K is the counting channeling factor from the ^{14}C-channel into the ^{3}H-channel.

in the writers' laboratory was used, it is shown that firm DNA-protein bonds can be tested and repaired in cultures of normal human fibroblasts treated with mustine.

EXPERIMENTAL METHOD

Normal human fibroblast cells were grown in medium containing 60% Eagle's medium, 30% lactalbumin hydrolysate, 10% bovine serum, thymidine-^{3}H (0.5 μCi/ml, specific activity 1.6 Ci/mmole), DL-valine-^{14}C (10 μCi/ml, specific activity 30 mCi/mmole), and L-leucine-^{14}C (2 μCi/ml, specific activity 200 mCi/mmole). The cells were grown for 34 h in medium with isotopes and then washed to remove the isotopes. A sterile solution of mustine was added to the medium 40 h after seeding, and the cells were incubated in the presence of the compound for 1 h at 37°C. Before, immediately after, and some time after incubation with the mutagen, samples of cells were taken from the monolayer by means of a spatula. To determine the relative proportions of radioactive isotopes incorporated into DNA and into protein firmly bound with DNA, the cells were sedimented from the medium by centrifugation for 5 min at 1000g. The residue was suspended in a cold solution of 6 M urea and 2.5 M NaCl in the presence of 0.01 M phosphate buffer, pH 6.9. The suspension was sonicated on an MSE (England) ultrasonic disintegrator, on the high-amplitude 4 program. Immediately before sonification 0.5 ml of homogenate at 2°C was applied to a hydroxyapatite (HAP; from Bio-Rad) column. The volume of the column was 0.1 ml. After application of the homogenate, 6 volumes of 0.01 M phosphate buffer containing 6 M urea and 2.5 M NaCl were passed through the column. The dissociated proteins were eluted at room temperature with 0.2 M phosphate buffer containing 6 M urea and 2.5 M NaCl, pH 6.9. The DNA was eluted with 0.4 M phosphate buffer, 6 M urea, and 2.5 M NaCl, pH 6.9. After elution the DNA was dialyzed against 10^{-3} M NaCl and 0.5 ml of the dialyzed solution was added to 9.5 ml of standard dioxane scintillator. The ^{14}C- and ^{3}H-activity was counted on an SL-30 counter.

Since some of the well-known and widely used methods of cloning cultures, vital staining, or accumulation of radioactive chromium are unsuitable for the determination of survival after prolonged culture of fibroblasts after treatment with mustine, in the present investigation a method consisting essentially as follows was used. The cells were grown for one cycle in a medium containing 0.2 μCi/ml thymidine-^{3}H, which was necessary to incorporate the radioactive label into the cell DNA. The cells were then transferred to medium not containing the radioactive isotope. A sterile physiological solution of mustine was added to the flask 40 h after transfer up to a final concentration of the mutagen of 2.5×10^{-5} M, and the contents were incubated at 37°C for 1 h. After washing the cells to remove mustine they were kept for 72 h in medium containing 2 μg/ml nonradioactive thymidine, which was necessary to prevent reutilization of the labeled DNA derived from the dead cells. The medium was then completely poured off and, after acid hydrolysis of the DNA contained in it, thymidine-^{3}H activity was counted. The monolayer of fibroblasts was covered with a fresh volume of medium which differed from the previous medium in containing deoxyribonuclease (DNase) I (from Sigma) in a concentration of 100 μg/ml and 10 μM $MgCl_2$. The cultures of fibroblasts were incubated in this medium at 20°C. Treatment with DNase led to removal of the labeled DNA from dying cells in the monolayer. After incubation for 2 h with DNase the medium was again completely poured off. After separation of the cell monolayer from the medium, acid hydrolysis with 5% HCl was carried out in both fractions and the radioactivity was then counted.

EXPERIMENTAL RESULTS

It will be clear from Fig. 1 that the DNA fraction eluted from the lysate of control cells on HAP contained a very low concentration of the ^{14}C label. After treatment of the fibroblasts with mustine the concentration of this label relative to ^{3}H increased significantly by 4-6 times and remained high at least during the first 5 h after removal of the mutagen. If the DNA fraction isolated from the experimental samples of cells were treated additionally with DNase I and again fractionated on HAP in the same dissociating system, as a result of degradation of the DNA the fraction eluted by a solution of 0.4 M phosphate buffer plus 2.5 M NaCl plus 6 M urea contained neither labeled protein nor labeled nucleic acids. An increase in the relative content of protein label in the DNA fraction after treatment of the fibroblasts with mustine cannot therefore be attributed to the presence of a class of proteins bound particularly firmly with HAP, which were not bound to DNA but could be eluted from the DNA fraction. As Fig. 1 shows, the ratio between protein label and DNA label in the fibroblasts 72 h after treatment with mutagen was identical with that in the control. This may have been due either to elimination of the DNA-protein cross-linkage or to death of the cells as a result of the formation of cross-linkages and detachment of the dying cells from the monolayer. However, a study of the survival of fibroblasts treated with mustine showed that during the 72 h after treatment with the mutagen activity of thymidine-^{3}H detectable in the incubation medium accounted for not more than 3-5% of the total activity of DNA in the previously labeled cells. After additional treatment with DNase I, about 3-5% of the total activity of DNA also was extracted from the cells. The same quantity of radioactive material was extracted after analogous washing of the control monolayer of cells untreated with mustine. It can be concluded from the equal yield of thymidine-^{3}H from the control and experimental cells that, under these conditions of incubation with the mutagen, at least 95% of the cells survived and were not detached from the monolayer during the first 72 h after removal of the mutagen. These results suggest that the decrease in the relative proportion of protein label in fibroblasts treated with mustine and kept for 72 h after removal of the agent resulted not in death of the cells, but in elimination of those DNA-protein cross-linkages which were preserved in the dissociating solvents during elution of the cell lysate through HAP.

Several methods whereby modification of DNA-protein bonds through the action of various physical and chemical mutagens can be studied have been published. To detect strengthening of DNA-protein bonds, mainly methods of gel-filtration [3], phenol- or chloroform-salt deprotienization in the presence of detergents [7, 11], or the study of the kinetics of filtration of the cell lysate through membrane filters [8, 9] have been used. In the case of phenol or chloroform deproteinization, DNA with firmly bound protein separates into two fractions. One remains in the aqueous phase in the soluble state [7, 11], the other passes into interphase. Most of the mutagens studied give rise to injuries of multiple character, which include not only modification of DNA-protein interaction, but also partial degradation of DNA, as a result of which DNA even if not bound with protein can interact with phenol and chloroform and can also pass into the interphase zone [2, 6]. It can thus be definitely asserted that the decrease in extractability of DNA is due entirely to the appearance of firmly bound protein. In studied of the kinetics of filtration through membrane filters, products held up by the filters also consist of a mixture of DNA, with intermolecular cross-linkages between the polynucleotide strands and DNA bound with protein. The use of either of these methods enables only the product that can be described as DNA with firmly bound protein to be identified in the damaged chromatin, whereas the method suggested in this paper enables the character of precisely this type of injury to be studied most fully. The problem of whether

repair of DNA-protein cross-linkages is possible has assumed great biological importance at the present time [1]. Moreover, data have been published to show that in certain inherited pathological conditions and, in particular, in xeroderma pigmentosum, the fibroblasts of the skin evidently cannot repair DNA-protein cross-linkages induced by UV-radiation [9]. The role of repair systems in the elimination of cross-linkages must therefore await further investigation.

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LIMIT OF CERTAIN REACTIONS OF THE CELL NUCLEUS IN OLD AGE AND IN CHRONIC CCl_4 POISONING

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The number of nucleoli and the structure of chromatin bound with the nucleolus (as reflected in the number of chromocenters) were investigated in two groups of mice: a control group and an experimental group receiving weekly injections of carbon tetrachloride for one year. During aging a gradual increase in the number of nucleoli and in the number of chromocenters bound with the nucleolus was observed in the control animals. During CCl_4 administration both these indices rapidly reached their maxima, which were the same as or a little higher than the values for the old animals. During subsequent development of the pathological process no further change took place in these indices.

KEY WORDS: nucleolus; chromatin; old age; carbon tetrachloride.

The similarity between age changes in the structure and function of organs and changes arising as a result of stress reactions [3, 7] or prolonged hyperfunction of an organ [2] has been described. The essential nature of the processes causing more rapid aging of organs affected by a pathological process has not yet been explained. The explanation given by most investigators, in its general form, can be reduced to the statement that the body possesses a certain reserve of adaptive capacity which becomes exhausted with age. In disease intensive mobilization of this reserve takes place, so that it becomes exhausted more rapidly.

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