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DNA DAMAGE: A CONSEQUENCE OF THE COMBINED EFFECT OF VIRUS INFECTION AND INCORPORATED RADIOACTIVE THYMIDINE

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SUMMARY : Infection of the human KB cell line with Frog Virus 3 brought about a partial degradation of radioactively labeled cell DNA as determined by alkaline sucrose sedimentation analysis. Further characterisation of this phenomenon revealed that degradation only occured when a sufficiently large amount of radioactive precursors was previously incorporated into the cell DNA. Therefore DNA damage appeared to be the result of a combined effect of incorporated radioisotopes and viral infection. It is suggested that the degradation of the radioactively labeled cell DNA could be a consequence of the inhibition of cell protein synthesis by Frog Virus 3 since treatment of the cells with cycloheximide also led to DNA degradation.

INTRODUCTION

Infection of cells with Frog Virus 3 (FV 3) rapidly reduces nucleic acid and protein syntheses (1, 2), the arrest of cellular metabolism finally resulting in cell death (3). This occurs, independently of any virus replication, either at supraoptimal temperature for virus growth, 37°C (4, 5), or with solubilized structural proteins (6, 7).

In a search for a better characterization of these phenomena, we considered the possibility that FV 3 may provoke DNA degradation. The integrity of cellular DNA molecules was studied by sedimentation of radioactively labeled DNA in alkaline sucrose gradients, a technique which is widely employed to detect single-strand breaks in DNA.

In this communication we report the effect of FV 3 infection on the sedimentation of labeled cellular DNA. Moreover, experiments have been carried out in order to determine whether the accumulation of DNA breaks can be attributed to the virus itself or whether the damage is related to the specific activity of cellular DNA and (or) to the diminution in cell metabolism.

Abbreviations : FV 3, Frog Virus 3 ; EDTA, ethylene dinitrilo-tetraacetic acid.

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MATERIAL AND METHODS

Human KB cells were grown in monolayers at 37° in a medium containing Lactalbumin, Yeast extract, Earle's salt and 5% calf serum. Frog Virus 3 was produced and purified as previously described (2).

To label the DNA, subconfluent KB cell cultures were incubated for 4, 16 or 24 h in minimum essential medium supplemented with 5% dialysed calf serum and containing different concentrations of radioactive thymidine as indicated in the legends, ([^{3}H] thymidine, 20 Ci/mmole ; [^{14}C] thymidine, 53 mCi/mmole). After the labeling period, cells were incubated overnight with fresh, unlabeled medium, to avoid any further incorporation of radioactive precursors during the experiment and to permit chain elongation.

To infect the prelabeled cells, culture medium was removed and the virus was allowed to adsorb at a multiplicity of 10 plaque forming units/cell for 1 hr at 37°C. Another series of cultures were mock-infected. The monolayers were then drained, growth medium was added and the cultures reincubated at 37°C. Alternately, for drug treatment, culture fluid was replaced with medium containing 5.10^{-3} M hydroxyurea, 100 µg/ml cycloheximide or 10 µg/ml actinomycin D.

After 3 hrs of infection or treatment with the drugs, cells were brought into suspension by incubation with trypsin, diluted rapidly with cold phosphate buffered saline and pelleted. They were resuspended in phosphate saline at a concentration of 10^5 cells/ml.

To analyse the sedimentation characteristics of the DNA, cells were lysed directly on the top of an alkaline sucrose gradient. A 30 ml linear (10-30% w/w) sucrose gradient was preformed over a 1.5 ml CsCl (60% w/v) cushion (all solutions contained 0.3 M NaOH, 0.01 M EDTA, 0.5 M NaCl), and overlayed with 2 ml of a lysing solution (0.5 M NaOH, 0.05 M EDTA). The CsCl cushion could be omitted since it did not modify the distribution between large and small DNA molecules although it reduced the dispersion in the gradient of the large DNA molecules. One milliliter of cell suspension (10^5 cells) was added on this gradient, left to stand overnight at 4°C and then centrifuged at 50 000 g for 7 hrs. Fractions were collected and the radioactivity determined after trichloroacetic acid precipitation. The results are expressed as the percentage of total counts recovered in the gradient.

The specific activities of the cells were determined as follows : 10^5 cells were precipitated with 10% trichloroacetic acid, the insoluble fraction was washed in the same solution and solubilized in scintillation liquid containing triton X 100. The radioactivity was measured and the counting efficiencies estimated by addition of internal standards ([^{14}C] toluene or [^{3}H] toluene). The specific activities were expressed as disintegrations per minute (dpm) per cell.

RESULTS AND DISCUSSION.

A DNA sedimentation pattern of [^{3}H] thymidine prelabeled cells, infected with FV 3 is shown in fig. 1a. For a more precise comparison, control cultures labeled with [^{14}C] thymidine were mixed with the infected cells and cosedimented. Undoubtedly, DNA fragments of low molecular weight appear after FV 3 infection. At first examination, this could be interpreted as a result of endonuclease activity. Two points would favor this hypothesis. First it is known that an endonuclease is closely associated with the virus particle (8). Second, FV 3 infection leads to cell death and DNA degradation has been described as an event precedent of, or concomitant with cell death (9). However, in another experiment,

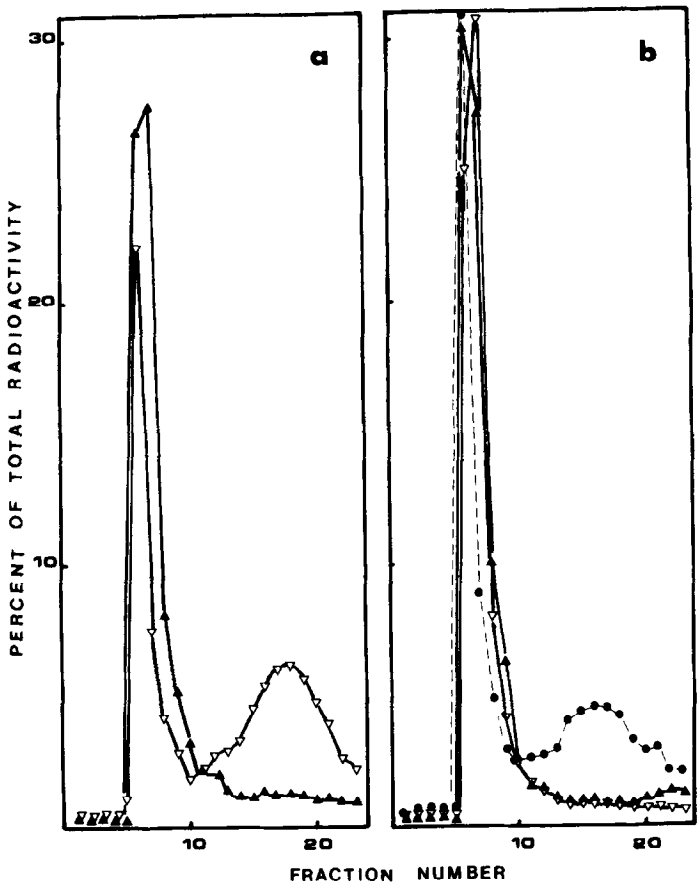

Fig. 1 : Cosedimentation profiles of FV 3 infected and uninfected cell DNA on an alkaline sucrose gradient. Cells were prelabeled for 4 hr with [^{3}H] thymidine (2 µCi/ml, incorporation per cell : 0.45 dpm) or with [^{14}C] thymidine(2 µCi/ml, incorporation per cell : 0.06 dpm)
(a) [^{3}H] infected cell DNA ▽ and [^{14}C] control cell DNA ▲
(b) [^{14}C] infected cell DNA ▲ and [^{3}H] control cell DNA ▽
Sedimentation pattern of infected cell DNA prelabeled for 24 h with 2 µCi of [^{14}C] thymidine (0.4 dpm/cell) (●) is represented in (b) ; the profile of uninfected cell DNA labeled in these conditions is identical to uninfected [^{3}H] DNA (0.45 dpm/cell) (▽). Sedimentation was from right to left.

to confirm this observation, [^{14}C] thymidine prelabeled cells were infected whereas [^{3}H] labeled cells were kept as a control. Surprisingly there was no difference in the DNA sedimentation profiles of uninfected and infected cells (fig. 1b). Thus a direct hydrolysis due to an endonuclease, either associated with the virus or induced in response to virus toxicity could be excluded as such an activity should be measurable on both [^{14}C] and [^{3}H] DNA. Rather the bimodal profile of infected cell DNA appears to be related to the presence of incorporated tritiated thymidine. However we must consider that as a result of the different specific activities of [^{3}H] thymidine and [^{14}C] thymidine

used, the amount of isotopes incorporated into DNA are respectively 0.45 and 0.06 disintegrations per minute per cell. By increasing the labeling time up to 24 h, a [^{14}C] thymidine incorporation of 0.4 dpm per cell can be reached, a value which is close to the one obtained with [^{3}H] thymidine in the previous conditions. Infection of these cells also led to the formation of small DNA molecules (fig. 1b). In consequence DNA degradation after FV 3 infection depends upon the amount of radioisotopes incorporated.

Incorporation of radioactive precursors into cell DNA has various effects on cells (for reviews see ref. 10 and 11), namely cell death (12, 13) and DNA strand breaks, this later phenomenon resulting directly from the β particle irradiation (14 - 16). It has been shown that strand breaks caused by isotope decay are rapidly rejoined (14, 17). Thus in cells where such damage occurs a continuous process of repair goes on, but only a limited number of damaged sites can be restored in a given interval of time (18).

We have found that, after FV 3 infection, the intrinsic ability of cells to repair DNA damage is diminished (results to be published). Thus some unrepaired breaks produced by internal irradiation may persist in the infected cells. When breaks are accumulated in the DNA of frozen cells, a shift of all DNA molecules towards smaller molecular weights is observed. However, if the repair capacity of uninfected cells, incubated at 37°, is overwhelmed by an increased isotope incorporation (5.6 dpm/cell), the DNA sedimentation profile mimics the bimodal pattern of infected cell DNA (fig. 2). This bimodal profile is observed even after a 24 labeling period to ensure that the isotopes were incorporated in most cells in an unifilar fashion. Different hypotheses may be put foreward to account for the bimodal degradation pattern of DNA in infected cells. Either an irregular repair occurs throughout the DNA as DNA accessibility can be restricted by the chromatine structure (18) or else differences exist in the cell population, for example cells at different stages of the cycle, the DNA being fragmented in only one more sensitive sub-population. It may still be a result of the combined toxic effects of the virus and the isotopes leading more rapidly to cell death.

In order to ascertain the involvement of various perturbations that occur in the infected cells such as i) the block in DNA replication ii) the inhibition of transcription iii) protein synthesis inhibition, comparison was made concerning the eventual role of inhibitors of these afore-mentioned functions in DNA breakdown.

DNA replication was inhibited in uninfected prelabeled cells by addition of hydroxyurea. It has been reported that transient DNA breaks are formed in hydroxyurea treated cells that are replicating their DNA, the appearance of these breaks being related to the depletion of the deoxynucleotide

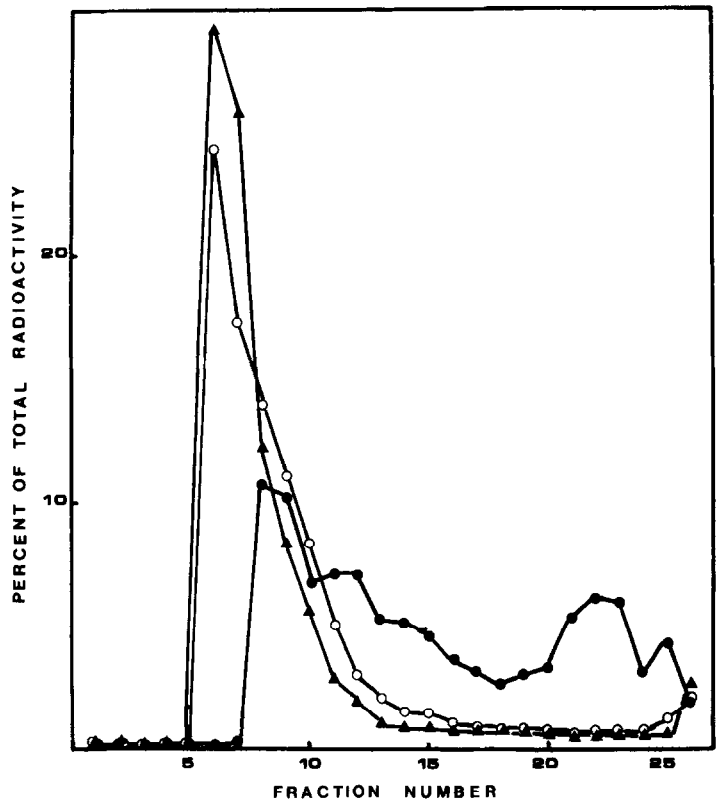

Fig. 2 : Effect of increasing incorporation of radioactive precursors on DNA sedimentation profile in alkaline sucrose gradients. Cells were labeled for 16 h with [^{3}H] thymidine : 2 µCi/ml (0.8 dpm/cell, O), 20 µCi/ml (5.6 dpm/cell, ●) or with [^{14}C] thymidine : 2 µCi/ml (0.3 dpm/cell, ▲). DNA was analyzed after a further incubation of 10 h in unlabeled medium. Sedimentation was from right to left.

pools (19). Although this phenomenon may be reflected by the dispersion of the peak under our experimental conditions (fig. 3a), it was only weakly expressed, possibly because, in the unsynchronized culture used, only a fraction of the cell population was in S phase or because the presence of CsCl at the bottom of the gradients could have masked small changes in the size of DNA. However, it is clear that the population of small DNA molecules observed in the infected cells does not appear here.

The involvment of a transcription block in the DNA damage observed is difficult to investigate since actinomycin D treatment, the commonly used inhibitor of RNA synthesis, results in a pronounced degradation of DNA (20) which can hardly be compared to that of infected cells ; it is not to be excluded that the complex formed between DNA and actinomycin can be recognized by excision enzymes involved in DNA repair leading to breaks independently of the presence of isotopes.

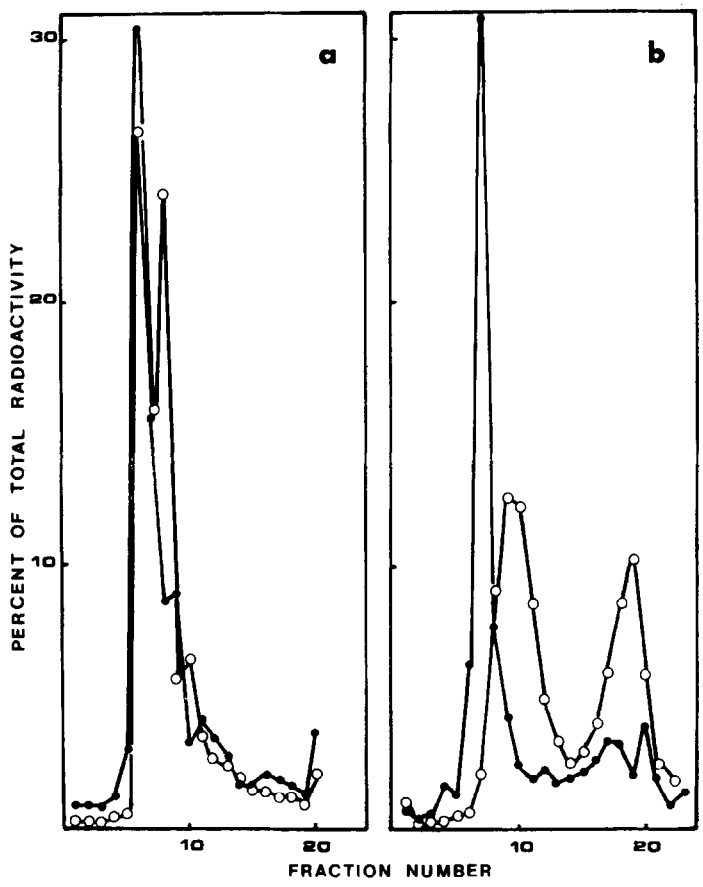

Fig. 3 : Sedimentation behavior of DNA from cells treated with inhibitors. Cells were prelabeled for 4 h with 2 μCi/ml [^{3}H] thymidine (0.45 dpm/cell) (a) Effect of a 3 h treatment with hydroxyurea (O), control cells (●). (b) Effect of the addition of cycloheximide for 3 h (O), followed by its removal, and a 3 h incubation in normal medium (●). Sedimentation was from right to left.

DNA degradation also occured in cells treated with cycloheximide as shown fig. 3b. After removal of the drug, when protein synthesis had resumed for 3 hr, DNA sedimentation was very similar to the pattern obtained from control cultures. This suggests that short-lived proteins are involved in maintaining the full length of isotopically labelled DNA molecules. Whether these proteins can regulate the degradation functions of others or participate directly in DNA repair is an open question. Although reports have shown that DNA damage can be repaired in the absence of protein synthesis (21, 22) it is also known that there is a variability in radiosensitivity from one cell line to another (13) which may suggest that the lifetime of the enzymes or the repair capacity are not identical in different cell lines.

In as much as an inhibition of protein synthesis results in the appearance of DNA breaks, a similar phenomenon can be expected in FV 3 infected cells where protein synthesis inhibition reached 50% of the control.

Most studies on the formation of DNA strand breakage by incorporated radioactive precursors have been carried out on non metabolizing frozen cells. Our experiments show that, in living cells incubated at optimal temperature, while incorporated radioactive thymidine does not induce any detectable modification of the sedimentation behavior of control cell DNA, a significant degradation of DNA chains is observed when cell metabolism is diminished either by a virus infection (FV 3) or by cycloheximide. Although we have not demonstrated the molecular basis for this DNA breakdown, this effect should be taken into consideration in the interpretation of the results when isotopes are involved.

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