CELLULAR ASSAY SYSTEMS

Moderator: Sanford Miller
Food and Drug Administration
Cell Damage by Near Ultraviolet Radiation: Role of DNA–Protein Cross-links

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ABSTRACT—Near UV radiation and hydrogen peroxide were found to act synergistically to damage bacteriophage (which contains only DNA and protein) and mammalian cells. DNA–protein cross-links are being considered as the critical molecular alteration that leads to biological damage.—JNCI 1982; 69:177–181.

Forty years ago, Stadler and Uber (1) discovered that the action spectrum for mutation in maize could be superimposed on the adsorption spectrum for DNA. However, when compared to the DNA adsorption spectrum, action spectra for inactivation of bacteria (2) or phage (3) have large deviations in the 300- to 400-nm region (NUV). This observation indicates that DNA is not the sole chromophore for biological damage by NUV radiation. In addition to action spectrum data, considerable evidence shows that the biological effects of NUV radiation are strikingly different from those of FUV radiation (200–300 nm) (4–7). As one of many illustrations of NUV and FUV differences, the bacterium Micrococcus radiodurans when compared to Escherichia coli is notoriously less sensitive to FUV radiation but is more sensitive than E. coli to NUV radiation [(8); Caimi P, Eisenstark A: Unpublished observations]. From these and numerous other observations, we conclude that the chromophore for FUV radiation, but not necessarily for NUV, is DNA.

In our research program, we are systematically examining the various biological effects of NUV radiation to identify specific chromophores, targets, molecular alterations, and repair mechanisms (both constitutive and induced). These effects are being tested in different environments (e.g., oxygen, nitrogen, or sensitizing agent).

During our studies, we discovered that tryptophan photoproducts (9, 10), especially H2O2 (3, 7, 11, 12), can act as sensitizers to NUV radiation; this report focuses on this synergistic effect of H2O2 and NUV radiation. We have irradiated bacterial cells, bacteriophage, mammalian cells, polypeptides, and amino acids in these studies.

We have been faced with a paradox that has confronted other investigators of NUV radiation; neither DNA nor protein has an absorption peak above 300 nm. Yet many biological effects are seen above the 300-nm wavelength. Even purified transforming DNA (13) and purified phage (3) can be inactivated by monochromatic NUV radiation above 300 nm.

MATERIALS AND METHODS

This report is derived from several distinct sets of experiments. Reference sources of materials and methods are: a) DNA–protein cross-links in phage T7 (12) and b) cross-links in L929 fibroblasts and B16 melanoma cells (14).

RESULTS AND DISCUSSION

DNA–Protein Cross-links via H2O2 Plus NUV Radiation Action on Phage T7

We have demonstrated that NUV radiation plus H2O2 synergistically inactivates phage T7 by producing DNA–protein cross-links, thus preventing injection of phage DNA into the host (12). Also, H2O2 enhances NUV radiation lethality and SS DNA breaks in bacteria (7), thus providing simple model systems for the study of these synergistic interactions. While action spectrum analysis indicates that synergism for phage is maximal at 340 nm (4), the frequency of SS DNA break induction cannot be correlated with the peak of cell killing (12); i.e., there is no apparent direct relationship between SS breaks and cell death. Therefore, we searched for other mechanisms of biological damage by H2O2 plus NUV radiation and found that the number of DNA–protein cross-links did correlate with the number of inactivated phage (12).

To identify cross-links, we appropriately treated double-labeled T7 phages (3H-labeled DNA and 14C-labeled protein) and sedimented them through CsCl block or step gradients (12). Alkaline sucrose gradients demonstrated that, while the overall pattern of protein separation was relatively unchanged, the DNA experienced a slight shift with H2O2 treatment and a considerable shift with NUV radiation plus H2O2 treatment. The fractions showing maximal 3H radioactivity were pooled, dialyzed, and layered on CsCl step gradients. After centrifugation, fractions were collected and counted.

The peak of 14C radioactivity (protein) cosedimenting with 3H radioactivity (DNA) demonstrated a substantial increase in the cosedimentation complex only when phages were treated with both NUV radiation and H2O2. These results are most easily explained by DNA–protein cross-linkage induced by NUV radiation plus H2O2 treatment of phage T7. This argument is strengthened by the small, but reproducible, decrease in buoyant density of the DNA with NUV radiation plus H2O2 treatment (1.687 g/cm3) versus

ABBREVIATIONS USED: FUV=far UV; NUV=near UV; PBS=phosphate-buffered saline; SS=single-strand; trans-Pt=trans-diaminedichloride platinum (II).


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that with the control (1.704 g/cm³), NUV radiation (1.702 g/cm³), or H₂O₂ (1.701 g/cm³) treatments. When NUV radiation plus H₂O₂-treated DNA fractions were pooled, dialyzed, digested with DNase I, and subjected to a second round of CsCl centrifugation, the liberated ¹⁴C counts banded at about 1.3 g/cm³, a position characteristic of protein.

This particular NUV radiation plus H₂O₂ treatment resulted in a surviving fraction of 5.9X10⁻⁶, which corresponds to 9.7 lethal hits per phage. (One lethal hit is the dose that yields 37% survival.) Under these conditions, 1.48% of phage protein was cross-linked to DNA or 0.153% per lethal hit. Because the total molecular weight of the protein per phage T₇ is 2.4X10⁵, the contribution of the molecule (38,000 daltons) of the major head protein is 38,000 (2.4X10⁵) or 0.158%.

The close agreement of these two values (0.153% vs. 0.158% theoretical cross-linked protein/lethal hit) suggests that one molecule of phage coat protein (gene 10) could cross-link with DNA per lethal event. Since this protein represents over 60% of the total protein in phage T₇, it would be present in sufficient quantities for the juxtaposition necessary for cross-linkage to occur between DNA and protein, particularly since cross-linkage probably has no preferred location with respect to the T₇ genome. That the cross-linkage is covalent was suggested by the fact that phages were lysed by either phenol or sodium dodecyl sulfate at high pH, which would have disrupted noncovalent bonds.

**STUDIES WITH MAMMALIAN CELLS**

In an effort to extrapolate the studies of DNA–protein cross-linking caused by NUV radiation and H₂O₂ to mammalian systems, we conducted experiments on established mouse cell lines, L929 fibroblasts and B16 melanoma cells. We used the DNA alkaline elution technique of Kohn (14) and Fornace and Kohn (15) for detection of DNA–protein cross-links. Alkaline elutions were run with internal reference cells (L1210 mouse leukemia) to facilitate cross-comparisons between treatments. The percentage of DNA retained on the filter when the L1210 DNA retention was 10% was termed “relative retention.” Prior to elution but after treatment, the L929 and B16 cells received 440 rad of X-ray to introduce SS breaks and promote DNA elution by tetra-propylammonium hydroxide-EDTA eluting solution (15). The L1210 reference cells received 220 rad of X-ray.

L929 cells were apparently uninfluenced by black-light-bulb NUV radiation in doses between 2X10⁵ J·m⁻² and 6X10⁵ J·m⁻². A slight replication delay was seen with 8X10⁵ J·m⁻² NUV radiation, but there was no long-term influence on cell cycle time. Irradiations were done with the cells immersed in PBS to obviate irradiated medium artifacts (16). A NUV radiation dose of 6X10⁵ J·m⁻² produced detectable DNA–protein cross-links as shown by the high filter retention of the NUV-radiation-treated cellular DNA over that of the control DNA (text-fig. 1). For these experiments, the control cells were incubated in PBS for 4.5 hours, which corresponded to the time that the irradiated cells were in PBS during the NUV radiation exposure. The filter retention was shown to be caused by DNA–protein cross-links (as opposed to DNA–DNA cross-links) because the treatment was sensitive to proteinase K digestion (text-fig. 1); whereas the NUV radiation and control curves are quite separate without proteinase K treatment, the curves are nearly coincident after proteinase K treatment. The NUV-radiation-induced DNA–protein cross-links were effectively repaired within 24 hours after treatment as shown by the return of the relative retention to control levels after a 24 hour post-treatment incubation in complete medium (text-fig. 2).

To compare NUV-radiation-induced DNA–protein cross-links to chemically induced DNA–protein cross-links, we treated L929 cells with trans-Pt, a putative DNA–protein cross-linking agent (17). L929 cells were exposed to trans-Pt for 1 hour in medium without serum; control cells were incubated for 1 hour in medium without serum and without added trans-Pt. Either a 200-µM or a 400-µM concentration of trans-Pt caused about a 50% loss of cells within 24 hours of treatment. However, 200 µM of trans-Pt-treated cells restored replication capacity by either 4 or 7 days after treatment, whereas 400 µM of trans-Pt-treated cells showed no replication capacity by 4 days and only slight replication capacity by 7 days after treatment. DNA alkaline elution data showed that a 400-µM trans-Pt treatment produced DNA relative retention that was only slightly higher than that produced by 6X10⁵ J·m⁻² NUV radiation (table 1). The DNA–protein nature of this retention was shown by the considerable loss of retention following proteinase K treatment. However, a 200-µM trans-Pt treatment produced DNA relative retention that was essentially the same as that of the controls (table 1).

The NUV radiation effects on B16 survival and replica-
tion are contradictory. We have shown and reported an inhibition of replication with a NUV radiation dose as low as $4 \times 10^5 \text{J.m}^{-2}$; however, at other times the replication data were essentially identical to those obtained with L929, i.e., no extended effects for NUV radiation. The variation in NUV radiation sensitivity seems to be most readily attributable to the variation in pigmentation in these melanoma cells that occurs with extended periods of time in culture (the less pigmented cells being more NUV radiation-sensitive); this aspect is being pursued. The B16 alkaline elution data presented were obtained from cultures that had little pigmentation and therefore exhibited the greatest sensitivity to NUV radiation. NUV radiation ($6 \times 10^5 \text{J.m}^{-2}$) induced DNA-protein cross-links in B16 cells, which as was observed in L929 cells were repaired within 24 hours after treatment (text-fig. 3). Proteinase K treatments confirmed the DNA-protein nature of this NUV radiation-induced DNA retention (data not shown). Thus B16 and L929 cells both exhibit similar DNA-protein damage and repair in response to NUV radiation.

Since NUV radiation plus $\text{H}_2\text{O}_2$ treatment has been shown to produce DNA-protein cross-linking in viruses (12), we were curious as to whether $\text{H}_2\text{O}_2$ might potentiate NUV radiation-induced DNA-protein cross-linking. Both L929 and B16 cells were extremely sensitive to $\text{H}_2\text{O}_2$ alone, with B16 showing slightly greater sensitivity. Coincident exposure to NUV radiation ($6 \times 10^5 \text{J.m}^{-2}$) and $\text{H}_2\text{O}_2$ ($1.5 \times 10^{-4} \text{M}$) did not significantly reduce cell survival. However, NUV radiation plus $\text{H}_2\text{O}_2$ did alter the DNA retention by alkaline elution (text-fig. 4). As before, NUV radiation alone showed relative retention greater than that of the control attributable to DNA-protein cross-links. $\text{H}_2\text{O}_2$ treatment alone showed less relative retention than controls; this result was anticipated since $\text{H}_2\text{O}_2$ is known to introduce SS breaks in DNA (18-20) and the alkaline elution of DNA is known to be accelerated by SS DNA breaks (21). The slightly lower retention by $\text{H}_2\text{O}_2$-treated B16 as compared to $\text{H}_2\text{O}_2$-treated L929 cells may indicate that the greater sensitivity of B16 to $\text{H}_2\text{O}_2$ is due to an increase in the number of SS breaks. Following the same reasoning, the even lower retention of NUV radiation plus $\text{H}_2\text{O}_2$-treated L929 DNA and B16 DNA may indicate that coincident NUV radiation treatment enhances SS breaking by $\text{H}_2\text{O}_2$ rather than increases DNA-protein cross-links.

<table>
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<th>Proteinase K treatment</th>
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<th>Treated cells, %</th>
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<td>trans-Pt$^c$</td>
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<td>24 hr</td>
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*Percentage of DNA retained on filter when L1210 DNA retention was 10%.

Incubation for 30 min in 0.5 mg proteinase K/ml while cells are on filter, after lysis, and prior to elution.

Dose=$6 \times 10^5 \text{J.m}^{-2}$.

Incubation for 4.5 hr in PBS.

Incubation for 1 hr in RPMI-1640 without serum.

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**Table 1.—Relative retention of L929 DNA**

**Text-figure 2.—Repair of NUV radiation-induced DNA-protein cross-links in L929 fibroblasts.** Percent of L929 DNA retained on filter when L1210 DNA retention is 10% (relative retention) vs. repair time. 0 time=termination of experiment at the end of treatment. 24 hr=replacement of PBS with complete medium and incubation for 24 hr before termination. NUV radiation=$6 \times 10^5 \text{J.m}^{-2}$.

**Text-figure 3.—NUV radiation-induced DNA-protein cross-links and their repair in B16 melanoma cells.** Percent of B16 DNA retained on filter when L1210 DNA retention is 10% (relative retention) vs. repair time. 0 time=termination of experiment at the end of treatment. 24 hr=replacement of PBS with complete medium and incubation for 24 hr before termination. NUV radiation=$6 \times 10^5 \text{J.m}^{-2}$.
Obviously, phage and mammalian cells respond differently to NUV radiation and H₂O₂ treatments. Whereas a NUV radiation plus H₂O₂ treatment creates DNA–protein crosslinks in T7 phage while a NUV radiation treatment alone does not, the reverse seems to be true in mammalian cells. Since the protein "environment" of the DNA in these cells varies widely, it is not surprising that a strong oxidizing agent like H₂O₂ should differentially alter the two systems.

REFERENCES


DISCUSSION

N. Krinsky: I wonder if you would care to comment on the apparent presence of the DNA–protein cross-linking?

A. Eisenstark: We know that some cross-linking will persist in untreated cells. We check this by using still another mouse cell line (L1210) labeled with tritiated thymidine for 2 days. These control cells are always added to irradiated cells labeled with $^{14}$C; thus we always do our experiments with two separate cell lines mixed together and we count the double labels. This provides an internal standard. Since the tritiated thymidine is always 5–10%, we choose a specific amount of $^3$H label to compare with the $^{14}$C. Our laboratory, as well as most other laboratories that use this elution technique, has chosen 10% as an internal standard; i.e., when 10% of the tritium remains on the filter, this is the figure that is compared with the $^{14}$C-treated cells.