H_2O_2 or cumene hydroperoxide, or 0.4 mM linoleic acid hydroperoxide and rat mammary parenchymal cell preparations in 50 mM potassium phosphate pH 7.4.

Results and discussion. Figure 1 shows the electron spin resonance (ESR) trace for the reaction of N-OH-AAF with rat mammary peroxidase in the presence of the peroxides H_2O_2, cumene hydroperoxide, and linoleic acid hydroperoxide. A g-value of 2.0063 and a splitting of 7.7–7.9 G characteristic of the nitroxyl free radical of N-OH-AAF were observed. The nitroxyl free radical is the predicted intermediate for the free radical oxidation of N-OH-AAF into NOF and N-OAc-AAF[10]. Formation of NOF and N-OAc-AAF and disappearance of N-OH-AAF were confirmed by thin-layer chromatography on Silica Gel F plates with a dichloromethane-acetone (85:5, v/v) solvent.

Figure 2 shows the effects of the antioxidants ascorbate, reduced glutathione and propyl gallate on the formation of nitroxyl radical. Antioxidant concentrations equal to H_2O_2 concentrations were used. Ascorbate, propyl gallate and reduced glutathione completely eliminated the nitroxyl free radical formed from N-OH-AAF. In the case of ascorbate and propyl gallate an ESR signal was observed as a result of the preferential oxidation of these compounds into their free radical forms. For ascorbate the observed signal is characteristic of the ascorbyl radical (A_H = 1.7 gauss), although for the particular spectrum presented modulation amplitude was large and the characteristic hyperfine structure of this radical was not resolved.

This study suggests that enzymes from a target cell are capable of activating the carcinogen N-OH-AAF into the more active N-OAc-AAF and NOF via a nitroxyl free radical intermediate in a mechanism analagous to that proposed for model system peroxidases. This reaction is inhibited by antioxidants including the physiological compounds, ascorbate and glutathione. Further purification and characterization of the rat mammary peroxidase and studies of the effects of antioxidant levels on the carcinogen activation are currently underway.

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DNA-dependent-DNA-polymerase: Possible limiting influence on cell reproduction during viral leukemogenesis*

J. P. OKunewick and P. G. Braunschweiger

Cancer Research Unit, Clinical Radiation Therapy Research Center, Allegheny General Hospital, 320 East North Avenue, Pittsburgh (PA 15212, USA), 2 May 1977

Summary. Evidence is presented that during viral leukemogenesis spleen cell nuclei show an increase in labelling index and mean grain count, that is not accompanied by any changes in the nuclear level of DNA-polymerase-α. It is suggested that polymerase production remains under the control of the normal cell mechanisms and the virus may affect cell proliferation by altering the primer-template levels.

Cancer development in both humans and animals is often characterized in the initial growth phase by a population of rapidly proliferating cells and in the later growth phase by a large compartment of slow growing or resting, G_0, cells[1]. Previous studies in our laboratories have shown this also to be the case in mice given Rauscher leukemia virus, RLV[2–4], for which the hematopoietic stem cell has been identified as a leukemia virus target cell[5–7]. In these mice, even though the first response to the virus is to increase the number of DNA synthesizing (S-phase) cells[3,4], measurement of the splenic stem cell G_0 compartment at the 14th day after virus administration has shown it to be 20 times the normal size for a non-leukemic control[2]. Since at the same time, and at still later disease stages, Rauscher leukemic mice are also known to have massive serum virus titers[8], it can be inferred that although RLV does initially increase the number of proliferating S-phase stem cells it cannot of itself maintain an increased rate of proliferation in the target stem cells, even at these high virus titers. Hence, the possibility is suggested that there may be a limiting necessary factor for DNA synthesis, even in the leukemic cell, which cannot be influenced by the virus.

To further investigate this possibility in the Rauscher leukemia model we have utilized the primer-dependent-polymerase (PDP) assay technique recently developed by Schiffer et al.[9]. This technique allows one to study not only the changes in the fraction of proliferating cells during leukemogenesis through DNA labelling, but further to discriminate within the labelled populations as to whether the changes in labelling are a consequence of changes within the cell nucleus in DNA-dependent-DNA-polymerase concentration, or in primer-template, or both[10].

Materials and methods. The animals used were female SJL/J mice injected with 50 $SED_{50/14}$ units of RLV[2–4] at 12 weeks of age. As controls normal, non-injected, mice selected from the same lots were used. To allow for correlation of the present studies with our previous G_0 studies[2] the mice were sacrificed 14 days after RLV injection. The spleens were removed, single cell supensions prepared in Hank's solution supplemented with fetal calf serum, and dry film smears prepared on acid cleaned

* Acknowledgment. This work was supported in part by NIH-NCI grant No. 1-PO_2-CA-10438 and by USERDA contract No. E (11-1) 3097, P.G.B. was also supported by contract NCI-CB-43899.

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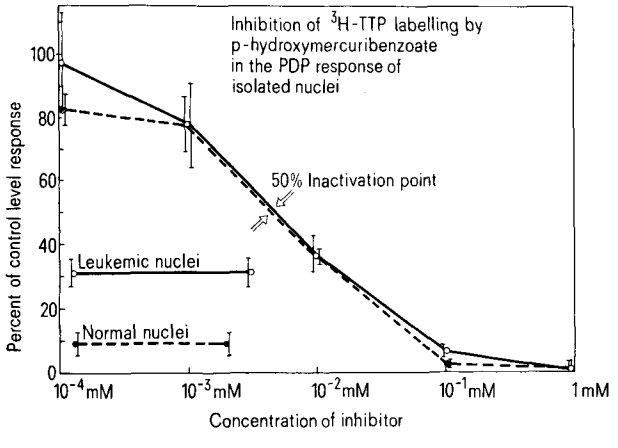
slides. The unfixed preparations were dipped into a 0.25% agar solution at 40 °C to strip away the cytoplasm, leaving the bare nuclei adherent to the slide, and air dried. Glass incubation chambers were afixed to the slides with paraffin, and 0.5 ml of incubation mixture containing tritiated thymidine triphosphate (^{3}HTTP) was added. The samples were then incubated and autoradiogrammed in the manner previously described, with positive ^{3}HTTP labelling indicating the simultaneous presence of primer-template in conjunction with DNA-polymerase-α within the cell nucleus[9–11]. Further evaluation of the relative levels of DNA-polymerase-α in the nuclei of leukemic spleen cells as compared to normal was accomplished by enzyme inactivation using para-hydroxymercuribenzoate (p-HMB), a potent sulfhydryl binding agent and inhibitor of DNA-polymerase-α[12]. For this varying concentrations of p-HMB, ranging from 1 mM to 10^{-4} mM, were added to the PDP incubation mixtures in otherwise duplicate slides at the beginning of the assay. DNA-polymerase-α inactivation was determined by the reduction in labelling achieved as a function of the p-HMB concentration.

Results. At sacrifice the leukemic mouse spleens were 4 times larger than normal, 395.5 ± 45.4 mg as compared to 93.2 ± 9.8 mg. The results for PDP labelling index and mean grain count for normal and leukemic nuclei incubated without p-HMB inhibitor are shown in the table. All nuclei were evaluated except those obviously belonging to differentiating myeloid cells. The results show that at 14 days after RLV injection, the inflection point for the growth phase Rauscher leukemia development[2,4], the overall PDP labelling index is 4.8 times that for normal control spleen cells, indicating that a greater proportion of leukemic cells than normal contain the 2 minimal requesites for PDP assay and DNA synthesis, primer and polymerase. Further, the mean grain count is increased by a factor of 2, suggesting that the concentration either of one or both of these is also increased on a per cell nucleus basis.

Comparison of PDP index parameters in spleen cell nuclei of normal and Rauscher leukemic SJL/J mice 14 days after virus infection

	Normal	Rauscher
Labelling index (± 1 SE)	13.09 ± 1.10	62.89 ± 3.55
Mean grain count (± 1 SE)	7.50 ± 0.24	15.36 ± 2.54



Extinction of PDP response in normal and viral leukemic cells as a function of concentration of the DNA-polymerase-α inhibitor, p-hydroxymercuribenzoate, in the PDP incubation medium. Loss in percent of control level reflects the reduction in mean grain count in terms of the percent value for either normal cell nuclei or leukemic cell nuclei not exposed to the inhibitor. Data indicates identical concentration of the polymerase in the normal and leukemic nuclei. The extinction curves are also similar in shape, within the limits of 2 SE, suggesting that the enzymes in the 2 types of nuclei are not significantly different.

The figure shows the inactivation of DNA-polymerase-α in normal and leukemic spleen cell nuclei as a function of p-HMB concentration. Inspection of the curves indicates that, proportionally, the polymerase inhibition for cells of the leukemic mice is similar to that of normal mice, with a virtually identical 50% inactivation point in terms of p-HMB concentration. The similarity of these curves suggests that in both cases the DNA-polymerase-α enzymes being evaluated were analogous, and that the average amount present per labelled cell nucleus was equivalent for the leukemic and normal spleen cells.

Discussion. In previous studies, Schiffer et al. have shown that the PDP technique yields a value for total labelling quite analogous to the growth fraction[9], and has an additional advantage in that one can directly evaluate the activity within the nuclei of the individual cells of 2 of the essential components which determine the capability for DNA synthesis, primer-template and DNA-polymerase-α[10]. Although by 14 days the fraction of Rauscher leukemic mouse stem cells in S-phase has returned to a value similar to that for non-leukemic mice[4], the present results show that the overall growth fraction for the general undifferentiated and differentiating spleen cell population is still substantially increased at this time. However, within that population, the average amount of DNA-polymerase-α per leukemic cell is similar to that in non-leukemic control spleen cells.

Schiffer et al.[10] have also reported that normal cell nuclei commonly contain a more than adequate amount of DNA-polymerase-α at the beginning of S-phase and have postulated available primer-template as a limiting factor governing the entrance of normal cells into DNA synthesis. This was based on their observation that addition of exogenous DNA template primer to the PDP system in normal cells resulted in a increased incorporation of ^{3}HTTP radiolabel. In light of this, the findings of the present study that DNA-polymerase-α levels in Rauscher leukemic nuclei are unchanged from the normal suggests by inference that the increase in the mean grain count, and hence radiolabel incorporation, in the PDP labelled leukemic cells may be the result of an opposite situation. Specifically, the action of the oncornavirus may result in leukemia-related excess of available primer-template per labelled nucleus, with the level of DNA-polymerase-α now becoming the limiting factor. This interpretation would be consistent with Temin's pro-virus hypothesis[13] and with other earlier reports on the production of both new linear and circular types of DNA by leukemia viruses[14,15] and their subsequent migration to the nucleus and incorporation into the host cell genome[16].

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