Screening for Deficits in DNA Repair by the Response of Irradiated Human Lymphocytes to Phytohemagglutinin

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SUMMARY

An assay has been developed to measure the ability of human lymphocytes to repair damage to DNA. In this assay, purified human lymphocytes are exposed to graded doses of radiation and then stimulated with phytohemagglutinin to undergo DNA replication. The rate of incorporation of thymidine in irradiated lymphocytes during the second and subsequent rounds of DNA replication is taken to be indicative of the ability of the cells to repair damage to DNA. In lymphocytes from normal individuals, X-irradiation with doses of 100 to 800 rads was found to inhibit phytohemagglutinin-stimulated thymidine incorporation proportionally to the dose of radiation without curtailing the induction of DNA polymerase.

The response to phytohemagglutinin of lymphocytes from a patient with xeroderma pigmentosum after exposure to graded doses of X-irradiation was found to be similar to that of the normal controls, whereas the response after ultraviolet irradiation was markedly impaired. In contrast, lymphocytes from patients with ataxia telangiectasia were hypersensitive to X-irradiation. The data on these clinical syndromes support the idea that this assay measures DNA repair and indicates the feasibility of using this method for screening individuals for genetic deficits in DNA repair.

INTRODUCTION

A deficiency in the ability of cells to repair damage to DNA could predispose individuals to somatic mutations that can lead to cancer (17, 19, 35). The classical example is that of XP, where a defect in DNA repair in skin fibroblasts has been correlated with a high incidence of skin cancer (6, 30). Similar, although less well-studied examples of the association of defective DNA repair to a high incidence of cancer include ataxia telangiectasia (24, 34) and Fanconi’s anemia (25). However, the above conditions are rare syndromes. In order to further evaluate the role of deficiency of DNA repair to oncogenesis, there is a need for a simple method to screen for such deficits in human populations. One of the methods that can be used for this purpose is based on the principle of measuring survival after damage to DNA as an index of DNA repair (22). It assumes that lack of adequate DNA repair prevents subsequent DNA replication and cell division. This has been used to measure DNA repair in mammalian cells by measuring the colony-forming ability of irradiated fibroblasts (34).

Adaptation of this test to human lymphocytes could provide a method for screening for deficits in DNA repair in human subjects since, compared to fibroblasts, these cells can be more readily obtained from different individuals. We have reasoned that the fraction of lymphocytes that survives in vitro X-ray or UV radiation could be quantified by measuring the rate of thymidine incorporation (DNA replication) in irradiated lymphocytes upon stimulation with PHA. In individuals with deficits in DNA repair, equal doses of radiation would be expected to cause greater inhibition of thymidine incorporation than in the normal controls. The validity of this approach was tested on lymphocytes from patients with XP or ataxia telangiectasia who are known to have deficiencies in DNA repair. The results presented in this paper are in accord with other studies of DNA repair in these patients, suggesting that the response of irradiated lymphocytes to PHA can be used to screen for deficits in DNA repair in human subjects.

MATERIALS AND METHODS

Culture of Lymphocytes. The peripheral blood was obtained from normal, healthy volunteers between 20 and 30 years old. Lymphocytes from peripheral blood were isolated by sedimentation on Ficoll-Hypaque gradients (4) or on nylon fiber columns (10). The cells separated by either method were diluted to 1 x 10^6 cells/ml in medium consisting of Eagle’s minimum essential medium (spinner modification) supplemented with 20% fetal calf serum, 2 mM L-glutamine, 100 units penicillin, and 100 μg streptomycin per ml, respectively. The cells were cultured in 1-ml aliquots in 16- x 125-mm Falcon plastic disposable culture tubes in humidified incubators at 37° with 5% CO₂ in air. PHA was added 4 hr after irradiation (25 μl to 1 x 10^6 cells in 1 ml). Similar results were obtained when PHA was added at any time up to 12 hr after irradiation.

Irradiation of Lymphocytes. The lymphocytes were irradiated within the culture tubes at room temperature with filtered 225 KVP X-rays from a General Electric Maximax X-ray machine. The radiation dose rate was determined with a Victoreen condenser R-meter and an f-factor of 0.945 rad/R. UV radiation was obtained from a 15-watt General Elec-

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4 The abbreviations used are: XP, xeroderma pigmentosum; PHA, phytohemagglutinin.

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tric germicidal lamp (86% emission at 254 nm). For UV irradiation, the cells (4 × 10⁶) were transferred to a 60- × 15-
mm Falcon tissue culture Petri dish to form a thin layer of cells (suspended in complete medium) that was irradiated
from the top with the lid off. The Petri dishes were gently rotated on a shaker during irradiation. The dose rate was
measured with an IL 1620 light meter that had been cali-
brated by malachite green actinometry (15). The total dose
of radiation was varied by controlling the time of exposure to UV and was corrected for the absorption by the medium
(12%). After irradiation, 1-ml aliquots of 1 × 10⁶ cells were
distributed to tissue culture tubes.

Measurement of the Rate of DNA Synthesis and DNA
Polymerase Activity. Cultures of lymphocytes were labeled with [³H]thymidine (6.7 Ci/mole, 2.5 μCi/culture) for 2 hr
before they were harvested. The incorporation of labeled
thymidine was stopped by adding 5 ml of ice-cold 0.15 M
KCl. The cells were pelleted and then lysed by repeated
freezing and thawing. DNA polymerase activity (both α and
β) in the cell lysate was determined in vitro with [α-³²P]dATP
as the labeled substrate and activated DNA as template (18).
This allows determination of the rate of DNA synthesis (³H
counts) and DNA polymerase activity (³²P counts) on the
same sample of cells.

Radioautographic Studies. Radioactive thymidine (as
above) was added 2 hr before the cultures were harvested.
Air-dried smears of lymphocytes were prepared according
to the technique of Hungerford (14). The slides were coated
with Kodak NTB2 emulsion and developed after 1 week.
The developed slides were stained with Wright’s stain. At least
1000 cells on each slide were scored for morphology and
labeling with [³H]thymidine.

RESULTS

Dose-Response Curve. The response of lymphocytes to
PHA after exposure to different doses of X-irradiation is
shown in Chart 1. In this experiment, the cells were irradiated
with X-ray doses between 25 and 1,600 rads. PHA was
added to the cultures 4 hr after irradiation, and the rate of
DNA replication was measured at 92 hr. In unirradiated,
PHA-stimulated cultures, the rate of [³H]thymidine incor-
poration was 32,500 ± 2,750 cpm/culture, and DNA polymer-
ase activity was 138 ± 3 pmoles [³²P]deoxythymidine mono-
phosphate incorporated per hr per culture. Irradiation of
lymphocytes with up to 100 rads had no significant effect
on the rate of thymidine incorporation. At doses between 200
and 800 rads, [³H]thymidine incorporation was diminished,
and the decrease was proportional to the X-ray dose.
Increasing the dose beyond 800 rads had no further inhibitory
effect. In contrast, the induction of DNA polymerase
remained unaffected by radiation up to 1,600 rads. Similar
results were obtained when cultures were harvested at
times between 72 and 96 hr after stimulation with PHA.
Irradiation by itself did not result in any detectable increase
in DNA polymerase activity or in thymidine incorporation
when assayed at 92 hr.

Effect of X-irradiation on the Course of PHA Stimulation.
The decrease in the rate of thymidine incorporation in irra-
diated cultures could represent a decrease in the rate of
DNA synthesis in all cells and/or a reduction in the number
of cells undergoing DNA replication. To determine the
kinetics of the response, cultures were harvested at different
times after the addition of PHA and processed for
[³H]thymidine incorporation and autoradiography. In unir-
radiated control cultures, DNA replication started between
20 and 44 hr after the addition of PHA, and the rate of
thymidine incorporation reached a maximum at 68 hr (Chart
2a). In this experiment, thymidine incorporation continued
at approximately the same rate until 140 hr. The appear-
ance and increase in the percentage of cells labeled with
[³H]thymidine followed a similar course (Chart 2b). At 44 hr,
32% of the cells were heavily labeled with [³H]thymidine,
and this number increased to 54.8% at 68 hr. At 92 and 114
hr, the values were 48.6 and 33.7%, respectively.

In lymphocytes irradiated with 400 rads the time course
for DNA synthesis was essentially similar to that observed
in unirradiated cultures except that the rate of thymidine in-
corporation was lower at all time points studied (Chart 2a).

Chart 1. The lymphocytes were separated on Ficoll-Hypaque gradients
and were irradiated at a dose rate of 130 rads/min. Four hr after irradiation,
PHA was added, and the cultures were harvested at 92 hr. All cultures were in
quintuplicate. The values of [³H]thymidine uptake (32,500 ± 2,750 cpm) and
DNA polymerase (138 ± 3 pmoles [³²P]deoxythymidine monophosphate/hr)
in unirradiated, PHA-stimulated lymphocyte cultures were taken as 100%. The
response of each irradiated culture was calculated individually as per-
centage of unirradiated control cultures, and the mean ± S.D. was calcu-
lated from these values. The line through the [³H]thymidine data points is
fitting by eye.

Chart 2. Cultures were harvested at different times after the addition of
PHA. The rate of [³H]thymidine uptake (by scintillation spectroscopy) and
percentage of cells engaged in DNA synthesis (by autoradiography) was
measured on parallel sets of cultures either unirradiated (controls) or ex-
posed to 400 rads (4 hr before PHA). All cells, irrespective of their size and
morphology, that had heavily incorporated [³H]thymidine grains were
included and considered to be in S phase. Bars, S.D.
In more detailed studies at early times (20 to 44 hr) it has been noted that the onset of DNA replication in irradiated lymphocytes is significantly delayed (data not presented here). After this initial delay, the rate of thymidine incorporation rapidly increased to near-normal levels. However, at later times thymidine incorporation in irradiated cells was progressively inhibited. Thus the rate of [3H]thymidine incorporation in irradiated cultures was (±S.D.) 60 ± 11% of controls at 44 hr, 69 ± 5% at 68 hr, 43 ± 5% at 92 hr, and 23 ± 5% at 114 hr. There was no difference in the percentage of cells engaged in DNA synthesis in the irradiated cultures compared to unirradiated cultures at 44 hr, being 72 and 73% of the controls at 68 and 92 hr, respectively, and 50% of the controls at 114 hr.

Response of Lymphocytes from Normal, Healthy Volunteers. The effect of PHA stimulation on irradiated lymphocytes is a fairly reproducible phenomenon. The degree of variation within replicate cultures is less than 10%, and repeat tests on lymphocytes from the same individual give similar results. However, the response of lymphocytes from different individuals varies. The results of studies on 16 normal healthy volunteers are shown in Chart 3. The lymphocytes from 2 of these individuals were relatively resistant to X-irradiation. This could be due to differences in the kinetics of the response to PHA. In the remaining 14, the rate of [3H]thymidine uptake in irradiated lymphocytes compared to unirradiated controls varied between 52 and 81% at 200 rads, 30 and 59% at 400 rads, and 18 and 37% at 800 rads. So far we have not seen marked sensitivity to X-irradiation in lymphocytes from any of the normal, healthy individuals.

Response of Lymphocytes from a Patient with XP. The response of lymphocytes from the patient with XP to PHA after in vitro irradiation with UV is summarized in Table 1. The patient was a 30-year-old female belonging to Group C of the XP DNA repair defect classification (30). The lymphocytes from this patient were markedly sensitive to UV irradiation. The rate of [3H]thymidine incorporation in lymphocytes irradiated with 10 ergs/sq mm was 8% of the unirradiated controls (Table 1). In comparison, in lymphocytes from the normal, healthy volunteer the rate of [3H]thymidine incorporation was 28% of the controls even after irradiation with an 8-fold higher dose (80 ergs/sq mm). On the other hand, the effect of X-irradiation on the lymphocytes from this patient was not as dramatic. At doses of 25 to 100 rads there was a 14 to 36% inhibition of thymidine incorporation, whereas the same doses did not significantly inhibit lymphocytes from normal healthy individuals (see Chart 1). At 400 and 800 rads the thymidine incorporation in lymphocytes from the patient was 37 ± 3% and 23 ± 2%, respectively. It was significantly lower (p = 0.005) than the response of lymphocytes from an age- and sex-matched normal healthy volunteer used as control in this experiment. However, the value of thymidine incorporation in irradiated lymphocytes from the patient with XP fall within the lower limits of the normal distribution (see Chart 3).

Response of Lymphocytes from Patients with Ataxia Telangiectasia. Lymphocytes from 2 siblings with ataxia telangiectasia were tested for their response to PHA after in vitro X-irradiation. The results are given in Table 2. After irradiation, inhibition of the rate of thymidine incorporation in PHA-stimulated, irradiated lymphocytes from these patients was much greater than that in the normal control at all doses. For example, after irradiation with 100 rads, the rate of [3H]thymidine incorporation in lymphocytes from patients

<table>
<thead>
<tr>
<th>Treatment (ergs/sq mm)</th>
<th>Patient with XP</th>
<th>Normal control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No radiation</td>
<td>100±8</td>
<td>100±8</td>
</tr>
<tr>
<td>10</td>
<td>8 ± 3</td>
<td>NT±c</td>
</tr>
<tr>
<td>20</td>
<td>4 ± 3</td>
<td>NT±c</td>
</tr>
<tr>
<td>40</td>
<td>1 ± 0.3±d</td>
<td>67 ± 16.0</td>
</tr>
<tr>
<td>80</td>
<td>1 ± 0.4±d</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>160</td>
<td>NT</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

± S.D. per culture.  

<table>
<thead>
<tr>
<th>Treatment (rads)</th>
<th>Normal control</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(E. T.)</td>
<td>(E. G.)</td>
<td>(D. G.)</td>
</tr>
<tr>
<td>No radiation</td>
<td>100±8</td>
<td>100±8</td>
<td>100±8</td>
</tr>
<tr>
<td>25</td>
<td>96 ± 4</td>
<td>85 ± 16±e</td>
<td>NT±c</td>
</tr>
<tr>
<td>50</td>
<td>92 ± 5</td>
<td>78 ± 10±f</td>
<td>75 ± 9±g</td>
</tr>
<tr>
<td>100</td>
<td>91 ± 6</td>
<td>62 ± 10±g</td>
<td>49 ± 7±j</td>
</tr>
<tr>
<td>200</td>
<td>66 ± 4</td>
<td>41 ± 6±k</td>
<td>28 ± 6±l</td>
</tr>
<tr>
<td>400</td>
<td>43 ± 4</td>
<td>22 ± 4±m</td>
<td>NT</td>
</tr>
<tr>
<td>800</td>
<td>22 ± 2</td>
<td>15 ± 2±n</td>
<td>NT</td>
</tr>
</tbody>
</table>

± S.D. per culture.  

* cpm/culture, 21,624 ± 2,098.  
* cpm/culture, 11,936 ± 2,333.  
* NT, not tested.  
* p = 0.005.
with ataxia telangiectasia was 56 and 49%, respectively compared to 91% in normal controls. More importantly, the response to PHA of irradiated lymphocytes from patients with ataxia telangiectasia was lower than that of any normal, healthy individual studied so far (see Chart 3). However, as in lymphocytes from normal individuals, the induction of DNA polymerase was not inhibited (Table 3).

**Discussion**

The results of this study show that the replicative response of in vitro irradiated lymphocytes to PHA can provide an indirect measure of the ability of the cells to repair damage to DNA. In normal individuals the inhibition of the rate of \[^{3}H\]thymidine incorporation in irradiated lymphocytes upon stimulation with PHA was found to parallel the radiation dose. With the use of this assay, lymphocytes from patients with ataxia telangiectasia and XP were found to be unusually sensitive to the effects of X-irradiation and UV irradiation, respectively. These disease states are known to have deficits in DNA repair. Presumably, if the DNA of the cell is not adequately repaired, DNA replication is inhibited in subsequent generations.

The inhibition of the rate of thymidine incorporation in PHA-stimulated, irradiated lymphocytes could be due to either a decrease in the number of cells initially participating in DNA synthesis or a reduction in the rate of DNA synthesis in all cells as a result of damage to DNA. The results reported here show that irradiation did not interfere with the ability of the lymphocytes to respond to PHA. Detailed studies on the kinetics of response of irradiated lymphocytes to PHA have been done on lymphocytes from normal, healthy volunteers. There was no delay in the induction of DNA polymerase, and the 1st round of DNA replication in irradiated lymphocytes proceeded normally. At 44 hr after PHA, in lymphocytes from a normal healthy volunteer, the number of cells in S phase was almost identical in both the irradiated and unirradiated cultures. However, at later periods the effect of radiation damage became apparent, and the rate of \[^{3}H\]thymidine incorporation in irradiated lymphocytes was inhibited in proportion to the dose of radiation. By 68 and 92 hr after PHA stimulation, the percentage of cells participating in DNA replication in irradiated cultures was reduced to three-fourths of that in unirradiated cultures; by 116 hr it was 50%. This could be considered to represent postmitotic cell death (22). However, DNA polymerase activity in the irradiated and unirradiated cultures remained identical at all time points studied. This is unlike the effect of X-irradiation on the induction of DNA-synthesizing enzymes in partially hepatectomized rats (3). The induction of DNA polymerase in PHA-stimulated lymphocytes requires RNA and protein synthesis, and the half-life of the enzyme is about 6 hr (1). We have observed that, after irradiation with 400 rads, RNA and protein synthesis is not significantly diminished in PHA-stimulated lymphocytes (unpublished results). Thus, although the lymphocytes are not functionally dead, they are unable to participate in subsequent rounds of DNA replication. Also, the decrease in the rate of thymidine incorporation was greater than the decrease in the number of cells in S phase, suggesting that even those cells engaged in DNA synthesis may not be replicating DNA normally. The decrease in the rate of thymidine incorporation was not due to a decrease in DNA polymerase activity (Chart 1) or to alterations in nucleotide precursor pools (unpublished results).

The induction of DNA polymerase in PHA-stimulated lymphocytes has been shown to be closely correlated with the replication of DNA in these cells (18). Bertazzoni et al. (2) have suggested that an increase in DNA polymerase-α is specifically linked to DNA replication. However, others have found that all the polymerases (α, β, and γ) are equally stimulated with PHA (20). We have previously observed that a variety of agents (chloroquine, methyl prednisolone, colchicine, 5-fluorouracil deoxyribose, 1-β-D-arabinofuranosylcytosine) coordinately inhibit the induction of DNA polymerase and DNA replication (unpublished observations). In contrast, X-irradiation does not inhibit the induction of DNA polymerase even though it inhibits DNA replication. This lack of parallel between DNA polymerase activity and DNA synthesis demonstrates for the 1st time that the inhibition of DNA replication does not reciprocally shut off the synthesis of DNA polymerase.

The sensitivity of lymphocytes from the patient with XP was much more marked to UV irradiation than to X-irradiation. This differential defect in DNA repair was first observed by Cleaver (7) in skin fibroblasts from these patients. Subsequent work has shown that XP cells are also differentially sensitive to different chemicals (8, 27, 28, 32, 33). It should also be emphasized that patients with XP exhibit a considerable degree of genetic heterogeneity. On the basis of complementation studies, at least 5 distinct groups (Groups A to E) of excision repair defects have been identified (16). In addition, a 6th type has been designated as "variant form" where the excision repair is normal but the cells are defective in postreplication repair. The XP patient included in this study belonged to Group C of the complementation groups. The rate of DNA repair in the cells from patients of this group has been reported to be 10 to 25% of normal (30). It would be interesting to study whether the response of lymphocytes in the system reported here can identify individuals with more severe (Groups A and B) or less severe (Group D) repair defects.

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**Table 3**

Response of lymphocytes from a patient with ataxia telangiectasia to PHA after in vitro X-irradiation

<table>
<thead>
<tr>
<th>Treatment (rads)</th>
<th>Normal control (E.T.) % DNA polymerase activity (mean ± S.D.)</th>
<th>Patient 1 (E.G.)</th>
<th>Patient 2 (D.G.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No radiation</td>
<td>100 ± 0.025-0.05</td>
<td>97 ± 5*</td>
<td>NT</td>
</tr>
<tr>
<td>25</td>
<td>100 ± 4</td>
<td>97 ± 5*</td>
<td>NT</td>
</tr>
<tr>
<td>50</td>
<td>102 ± 6</td>
<td>100 ± 8</td>
<td>94 ± 11*</td>
</tr>
<tr>
<td>100</td>
<td>103 ± 4</td>
<td>98 ± 5*</td>
<td>84 ± 5*</td>
</tr>
<tr>
<td>200</td>
<td>98 ± 5</td>
<td>98 ± 5</td>
<td>94 ± 13</td>
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<tr>
<td>400</td>
<td>93 ± 3</td>
<td>94 ± 6</td>
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</tr>
<tr>
<td>800</td>
<td>91 ± 5</td>
<td>90 ± 4</td>
<td>NT</td>
</tr>
</tbody>
</table>

* p = 0.025-0.05.  
† NT, not tested.  
‡ p = 0.005.
A deficit of DNA repair in patients with ataxia telangiectasia has recently been described. Although earlier cytogenetic studies had shown that in vitro X-irradiation of cells from patients with ataxia telangiectasia produced a high frequency of chromosomal aberrations, there was no clear evidence of defective DNA repair in these patients (24). Vincent et al. (36) did not find any difference in the kinetics and extent of strand repair after X-irradiation in skin fibroblasts from patients with ataxia telangiectasia and normal controls. However, the survival of skin fibroblasts after X-irradiation from these patients was found to be significantly reduced (34). It has recently been reported by Paterson et al. (24) that the rate of disappearance of $\gamma$-endonuclease-susceptible sites in the DNA of cells from patients with ataxia telangiectasia is slower than in normal controls. Furthermore, "repair replication" in cells from patients with ataxia telangiectasia was found to be about half that of normal controls (24). The present demonstration of reduced [H]$^3$thymidine incorporation in X-irradiated lymphocytes from patients with ataxia telangiectasia provides evidence for defective DNA repair in a 2nd cell type in these patients. It should be mentioned that there may be heterogeneity in the ability of patients with ataxia telangiectasia to repair DNA damage (13).

Our results indicate that the response of irradiated lymphocytes to PHA can be used to screen for deficits in DNA repair. Other methods for measuring DNA repair (9) include measurement of: (a) unscheduled DNA synthesis (23, 26), (b) excision of UV-induced thymidine dimers (5, 29), (c) nonsensonservative DNA synthesis (repair replication) (12, 23), (d) postreplication repair (31), (e) rejoining of single-strand breaks after X-irradiation (21), (f) host cell reactivation of irradiated viruses (11), and (g) survival of fibroblasts by colony formation. Except for the measurement of unscheduled DNA synthesis, most of these methods are complex and time consuming. In comparison, the measurement of thymidine incorporation in irradiated lymphocytes stimulated with PHA is a relatively simple and sensitive test. Furthermore, compared to unscheduled DNA synthesis, the method reported in this paper offers the advantage of detecting the deficiencies in the ability to repair X-ray-induced damage to DNA as well. This test is being used for screening for deficits of DNA repair in human populations.

REFERENCES