[CANCER RESEARCH 41, 3973-3978, October 1981]
0008-5472/81/0041-0000$02.00

DNA Replication in X-Irradiated Human Lymphocytes[1]

Shyam S. Agarwal,[2] Edward J. Katz, Awtar Krishan,[3] and Lawrence A. Loeb[4]

The Joseph Gottstein Memorial Laboratory, Department of Pathology, School of Medicine, University of Washington, Seattle, Washington 98195, and The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

ABSTRACT

Human peripheral blood lymphocytes are well-differentiated cells. Ordinarily, they do not divide and are considered to be in the G_0 stage of the cell cycle. These cells can be stimulated to undergo DNA replication in culture by mitogens such as phytohemagglutinin. In the present study, we have examined cellular and biochemical events that occur after exposure of lymphocytes to X-irradiation. Irradiation with up to 100 rads, prior to stimulation with phytohemagglutinin, did not interfere with DNA replication. At later periods, DNA replication was inhibited proportionally to the amount of radiation. In comparison to DNA synthesis, the effect of X-irradiation on RNA and protein synthesis in phytohemagglutinin-stimulated lymphocytes was less marked. Furthermore, X-rays did not inhibit either the induction or the continual synthesis of DNA polymerase-α or -β in response to phytohemagglutinin. Kinetic studies with different nucleotide substrates suggest that cellular pools of nucleotides are not significantly altered by X-irradiation. Thus, the inhibition of DNA synthesis in irradiated cells is likely to be due to damage to the cellular DNA template. The inhibition of DNA synthesis was accompanied by accumulation of cells in the G_2 and M stages of the cell cycle, suggesting that inhibition of DNA replication by X-irradiation is a postmitotic event.

INTRODUCTION

Peripheral blood lymphocytes are essentially nondividing cells and are considered to be in the G_0 phase of the cell cycle. They can be induced to undergo DNA replication upon stimulation with a variety of agents (22). We have reported that DNA replication, quantitated by radioactive thymidine incorporation in irradiated lymphocytes after stimulation with PHA[5], may be used to screen for abnormal response to radiation in human populations (1). The observation that thymidine incorporation in PHA-stimulated lymphocytes from patients with xeroderma pigmentosum and ataxia telangiectasia was significantly low compared to normal controls, after UV-irradiation and X-irradiation, respectively, suggests that the assay can detect radiosensitivity and, perhaps, defective DNA repair. Since peripheral blood is easily obtainable and thymidine incorporation during DNA replication is readily quantitatable, this system offers advantages in screening large populations for abnormal response to radiation. Also, studies on lymphocytes do not require prolonged culture *in vitro* prior to an experiment, thus eliminating the possible effects of selection *in vitro*.

In order to understand the sequence of events in the lymphocyte assay system, we have investigated the molecular and cellular events in the response of X-irradiated normal lymphocytes to PHA.

MATERIALS AND METHODS

Culture and Irradiation of Lymphocytes. Heparinized peripheral blood was obtained from normal healthy volunteers. Lymphocytes were separated by sedimentation on a Ficoll-Hypaque gradient by a modified method of Boyum (10). Lymphocyte cultures were stimulated with PHA as described previously (2). Where indicated, lymphocytes in culture tubes were irradiated with filtered 225 kVp X-rays from a General Electric Maximar X-ray machine (1).

Isotope Incorporation and DNA Polymerase Activity. Cultures of lymphocytes containing 10^6 cells in 1 ml were labeled with [^{3}H]thymidine (2.5 μCi; 6.7 Ci/mmol), [^{3}H]deoxycytidine (2.5 μCi; 38.1 Ci/mmol), [^{3}H]uridine (2.5 μCi; 25 Ci/mmol), or an algal [^{3}H]hydrolysate (5.0 μCi) for 2 hr before harvesting. Incorporation was stopped by adding 5 ml of ice-cold 0.15 M KCl, and the cells were washed 2 times by centrifugation in 5 ml 0.15 M KCl. To the cell pellet, 0.1 ml of a solution containing 20% glycerol, 0.02 M Tris-maleate buffer (pH 7.8), 1 mM potassium EDTA, and 1 mM dithrothreitol was added, and the suspension was frozen at −70° until being assayed for DNA polymerase activity. DNA polymerase activity (both -α and -β) in the cell lysate was determined *in vitro* with [α-^{32}P]TTP as the labeled substrate and activated DNA as the added template (24). This assay allows determination of the rate of DNA synthesis using [^{3}H]thymidine as a labeled precursor (^{3}H counts) and DNA polymerase activity (^{32}P counts) on the same sample of cells.

Studies on Cell Morphology. Morphological changes in PHA-stimulated lymphocytes after staining with Wright's stain were quantitated according to the technique of Hungerford (19). For radioautographic studies, cultures were labeled with [^{3}H]thymidine for the terminal 2 hr, and the radioautograms were prepared by coating the slides with Kodak NTB 2 emulsion and developing them after 1 week. At least 1000 cells from each slide were scored.

Cytofluorometry. At indicated times, cultures of PHA-stimulated lymphocytes were centrifuged (150 × *g*; 5 min), and the cell pellets were suspended in a propidium iodide/hypotonic citrate solution (21). The stained samples were analyzed on a cytofluorograph fitted with a pulse-height analyzer and X-Y recorder (22). For quantitation of the cytofluorograms, the area under each curve of the histogram was carefully cut out. For demarcating the area under the curve, a perpendicular line was dropped from the deepest portion of each trough. The cut pieces were coded and weighed on an analytical balance. The percentage of cells under each peak was determined as a fraction of the weight of the paper compared to the weight of the total histogram.

[1] This work was supported by Grants CA-06551, RR-05539, CA-11524, CA-12818, CA-15139, and CA-06927, awarded by the National Cancer Institute, Department of Health, Education, and Welfare, and by an appropriation from the Commonwealth of Pennsylvania. Cytofluorometric studies were done at the cytokinetics laboratory of Sidney Farber Cancer Center which was supported by National Cancer Institute Grant GA-20008.

[2] Present address: Department of Medicine, King George's Medical College, Lucknow, India.

[3] Present address: Comprehensive Cancer Center for State of Florida, University of Miami Medical School, P. O. Box 520875, Biscayne Annex, Miami, Fla. 33152.

[4] To whom reprint requests should be addressed, at The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology SM-30, University of Washington School of Medicine, Seattle, Wash. 98195.

[5] The abbreviation used is: PHA, phytohemagglutinin.

Received March 10, 1980; accepted July 10, 1981.

RESULTS

Stimulation of human peripheral lymphocytes by PHA results in a 20- to 200-fold increase in the rate of thymidine incorporation and a parallel increase in DNA polymerase activity (2, 24, 25). The effect of *in vitro* irradiation of human lymphocytes on the induction of thymidine incorporation and DNA polymerase is shown in Chart 1. In this experiment, the lymphocytes were irradiated at Time zero with 25 to 6400 rads at a dose rate of 134.8 rads/min. PHA was added to the cultures 4 hr after irradiation, and the cultures were harvested 90 hr after the addition of PHA. In unirradiated PHA-stimulated lymphocytes, [^{3}H]thymidine incorporation during the last 2 hr in culture was 29,400 ± 1,860 (S.D.) cpm/culture, and the amount of DNA polymerase activity per culture was 183 ± 4.3 pmol [α-^{32}P]dTMP incorporated per culture per hr. In accord with our earlier observations, 25 to 100 rads did not significantly reduce DNA replication upon PHA stimulation. At higher doses (200 to 800 rads), thymidine incorporation was inhibited in proportion to the dose of radiation. In cultures irradiated with 200, 400, and 800 rads, [^{3}H]thymidine incorporation was 77.5 ± 6.0, 39.8 ± 3.3, and 21.9 ± 1.6%, respectively, of that of unirradiated control cultures. On the basis of 6 different experiments, the D_0 is estimated to be 340 rads. With increasing X-ray exposure, a low but significant amount of [^{3}H]thymidine incorporation was observed except after lymphocytes were irradiated with the largest dose, 6400 rads. This incorporation may occur in a radioresistant subpopulation of lymphocytes.

In contrast to the diminution of thymidine incorporation, the induction of DNA polymerase activity in irradiated lymphocytes was less inhibited by irradiation up to 3200 rads. In this experiment, the DNA polymerase activity in lymphocytes irradiated with 100 and 1600 rads was 80.6 ± 3.8 and 72.0 ± 3.2%, respectively, of the unirradiated PHA-stimulated controls. In other experiments, DNA polymerase activity was undiminished even after 1600 rads. However, the induction of DNA polymerase was reduced by 95% after exposure to 6200 rads.

Effects of Nucleotide Pools. The decrease in labeled thymidine incorporation in irradiated lymphocytes, without a parallel decrease in DNA polymerase activity, could be due to less rapid transport of thymidine across the cell membranes as a consequence of irradiation. To evaluate this possibility, the rate of incorporation of [^{3}H]thymidine and [^{3}H]deoxycytidine after PHA stimulation of irradiated lymphocytes was compared. In lymphocytes irradiated with up to 400 rads, deoxycytidine incorporation was diminished in parallel to thymidine incorporation, suggesting that thymidine transport is not specifically diminished by X-irradiation. An alternative can be considered. The inhibition of thymidine incorporation is specious and results from an increase in the intracellular or extracellular concentrations of unlabeled TTP's or their precursors, mediated by DNA breakdown after X-irradiation. Thus, the amount of thymidine incorporated might be undiminished, but its specific activity would be less due to an increased amount of an unlabeled precursor as a result of X-irradiation. If this were so, the dilution of [^{3}H]thymidine with nonradioactive thymidine would differentially affect the amount of radioactive thymidine incorporated into DNA in unirradiated and irradiated cultures. The results of such an experiment are shown in Chart 2. In the presence of 0.003 μmol of [^{3}H]thymidine, incorporation in unirradiated lymphocytes was 32,818 cpm/2 hr/culture, while in lymphocytes irradiated with 400 rads, it was 17,772 cpm/2 hr/culture. The addition of 100- and 1000-fold excess of nonradioactive thymidine reduced the incorporation of labeled thymidine equally in both the unirradiated and irradiated lymphocytes. The parallel reduction by graded amounts of unlabeled thymidine suggests that the thymidine nucleotide pools are similar in irradiated and unirradiated cultures and changes in these pools do

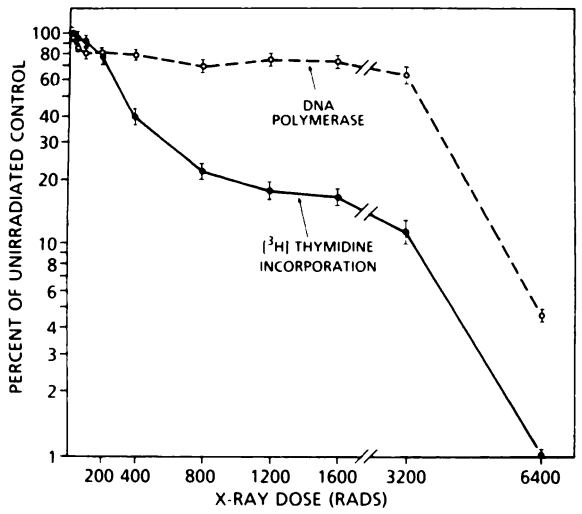

Chart 1. Effect of X-irradiation on lymphocyte stimulation by PHA. Quintuplicate cultures of isolated lymphocytes were irradiated with different doses of X-rays at Time zero. Four hr after irradiation, the cells were stimulated with PHA. At 90 hr after stimulation with PHA, the rate of thymidine incorporation was measured by labeling with [^{3}H]thymidine for the last 2 hr in culture. DNA polymerase activity was subsequently measured on the lysate from the same lymphocytes as described in "Materials and Methods." One hundred % represents the rate of thymidine incorporation and the amount of DNA polymerase activity in the unirradiated lymphocytes. *Point*, mean of 25 independent computations of 5 control cultures and 5 cultures of lymphocytes irradiated with a given dose of X-rays; *bars*, S.D.

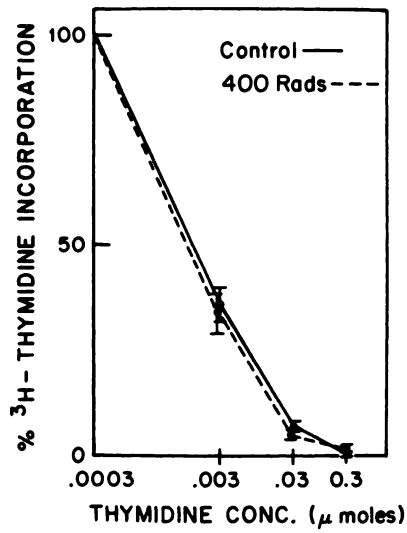

Chart 2. Effect of thymidine concentration. At 82 hr after stimulation of lymphocytes with PHA, the rate of [^{3}H]thymidine incorporation was measured by pulse labeling with [^{3}H]thymidine during the last 2 hr in culture in the presence of increasing concentrations of nonradioactive thymidine. The amount of thymidine incorporation in the absence of added unlabeled thymidine was taken as 100%. Corresponding values for unirradiated and irradiated lymphocytes were 32,818 ± 3,250 and 17,772 ± 2,336 cpm/culture, respectively. *Point*, mean of 4 cultures; *bars*, S.D.

not account for the inhibition of thymidine incorporation in irradiated cultures.

Effect of X-Irradiated Medium on the PHA Response. In the previous experiments, lymphocytes were irradiated in culture tubes and remained in the same culture medium in which they were suspended at the time of irradiation. Theoretically, X-rays could produce changes in the medium that inhibit the response of lymphocytes to PHA. To investigate this possibility, lymphocytes were separated after exposure to X-rays and resuspended in new unirradiated medium, prior to PHA stimulation (Table 1). In a reciprocal experiment, unirradiated lymphocytes were resuspended in irradiated medium. In neither case was the effect of X-irradiation on thymidine incorporation modified by the culture medium. Thus, the changes observed in the irradiated cultures are directly due to the effect of X-rays on the cells themselves.

Effect of X-Irradiation on DNA Polymerase-α and -β. The assay for DNA polymerase in PHA-stimulated lymphocytes mainly measures the DNA polymerase-α (29), the major activity in eukaryotic cells and one believed to be involved in DNA replication. DNA polymerase-β is a distinct molecular species that has been hypothesized to function in DNA repair (8). The latter enzyme is resistant to *N*-ethylmaleimide and high salt. Measurements of DNA polymerase in crude lymphocyte lysates in the presence of 10 mM *N*-ethylmaleimide and/or 0.2 M KCl was, therefore, considered to represent the activity of DNA polymerase-β (13). Under these conditions, the remaining activity, which is presumably DNA polymerase-β, was 10 to 20% of the total polymerase activity in reasonable agreement with the reports of others (8, 29). X-Irradiation without PHA did not stimulate either DNA polymerase-α or -β (results not shown). Measurement of total DNA polymerase activity and of DNA polymerase-β in lymphocytes irradiated with different doses of X-rays and then stimulated with PHA are given in Chart 3. Neither of these activities was markedly inhibited by prior exposure of lymphocytes to X-irradiation. Thus, the lack of effect of X-rays on the induction of DNA polymerase activity is not limited to DNA polymerase-α.

Relationship of RNA and Protein Synthesis to DNA Polymerase Activity in Irradiated Lymphocytes. We have shown previously that the increase of DNA polymerase activity after PHA stimulation requires the continued synthesis of RNA and proteins (2). To investigate the effect of X-irradiation on these parameters, the rate of incorporation of labeled uridine and amino acids was measured (Chart 4). There was a dose-dependent inhibition of RNA and protein synthesis in irradiated

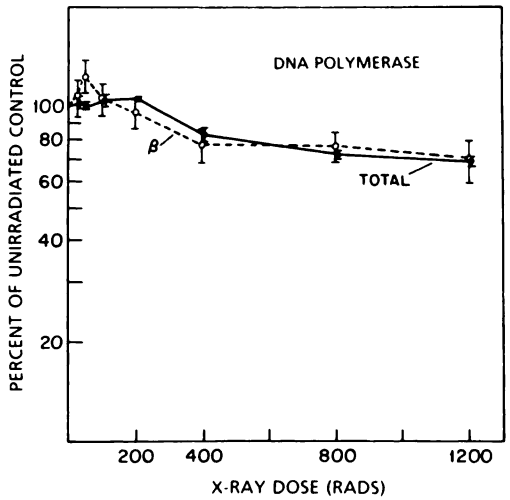

Chart 3. Effect of X-irradiation on DNA polymerase-β. Total DNA polymerase activity was measured after irradiation and stimulation with PHA, as described in "Materials and Methods." For the estimation of polymerase-β activity, assays were carried out in the presence of 10 mM *N*-ethylmaleimide.

Table 1

Effect of X-irradiated culture medium on response of lymphocytes to PHA

Lymphocytes were cultured in 16- x 125-mm plastic tubes and [^{3}H]thymidine incorporation was measured as described in "Materials and Methods." In Experiment A, the medium was changed at the indicated time after isolating the cells by centrifugation. In cultures where the medium was changed at Time zero, PHA was added 4 hr later. In cultures where the medium was changed at 12 hr, the lymphocytes were stimulated with PHA at Time zero and no additional PHA was added. In Experiment B, the cells were separated by centrifugation at 500 × *g* for 10 min. Thereafter, the cells were suspended in culture medium which was either unirradiated or irradiated 1 hr previously with 400 rads. PHA was added and [^{3}H]thymidine incorporation and DNA polymerase activity were measured at 66 hr.

	[^{3}H]Thymidine incorporation		
		Cells irradiated with 400 rads	
Experiment A	Unirradiated control cells (cpm/culture)	cpm/culture	% of control
Medium change			
None	27,243 ± 3,031[a]	7,175 ± 1,169	26.6 ± 4.9
Time zero	18,853 ± 778	6,012 ± 583	31.9 ± 3.0
12 hr after PHA	28,138 ± 2,962	9,174 ± 1,414	32.8 ± 5.6

	Unirradiated cells resuspended in			
	Unirradiated medium		Medium irradiated with 400 rads	
Experiment B	[^{3}H]Thymidine incorporation (cpm/culture)	DNA polymerase activity (pmol [α-^{32}P]dTMP incorporated/hr/culture)	[^{3}H]Thymidine incorporation (cpm/culture)	DNA polymerase activity (pmol [α-^{32}P]dTMP incorporated/hr/culture)
	16,293 ± 1,138	160.4 ± 5.9	15,205 ± 1,925	161.8 ± 6.3

[a] Mean ± S.D. of triplicate cultures.

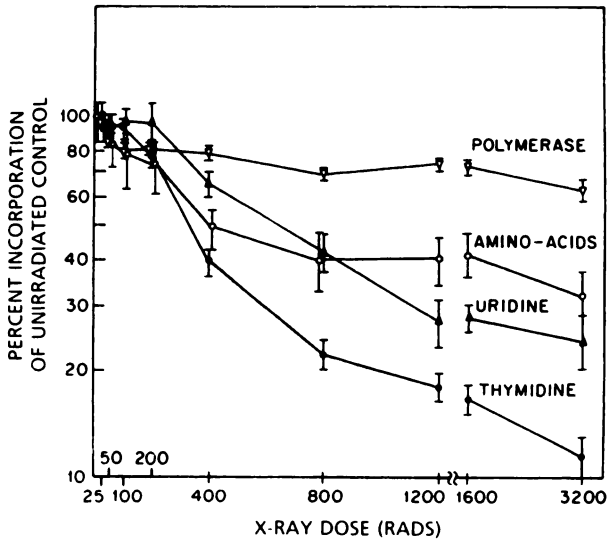

Chart 4. Effect of irradiation of RNA and protein synthesis. Sets of cultures were pulse labeled with [^{3}H]thymidine or [^{3}H]uridine or ^{3}H-amino acids 82 hr after PHA stimulation. The results are given as the percentage of incorporation of each precursor in unirradiated PHA-stimulated lymphocytes. DNA polymerase activity was determined in the cells used to measure thymidine incorporation (see "Materials and Methods").

lymphocytes, but the inhibition was not as pronounced as that of DNA synthesis. In irradiated lymphocytes, 30 to 40% of [^{3}H]uridine incorporation and ^{3}H-amino acid incorporation persisted after exposure to 3200 rads. The difference between polymerase activity and amino acid incorporation suggests that the increase in DNA polymerase activity is less inhibited by X-irradiation than the average rate of synthesis of other proteins.

Kinetics of Response to PHA after Irradiation. PHA activates sequential metabolic changes in lymphocytes culminating in DNA replication and cell division (2, 23). We have shown previously that the initiation of DNA synthesis and increased DNA polymerase activity are not detectable until 14 hr after the addition of PHA, and both reach a maximum by 3 to 5 days (2). In order to monitor the effect of X-irradiation on selected biochemical parameters, irradiated lymphocyte cultures were harvested at 6, 16, 24, 30, 40, 64, and 88 hr after PHA addition. At each time, the rate of incorporation of thymidine, uridine, and amino acids, as well as DNA polymerase activity, was measured (Chart 5). In this experiment, the increased incorporation of thymidine in both irradiated and unirradiated PHA-stimulated lymphocytes was first detected 24 hr after PHA stimulation. The increase in the rate of thymidine incorporation in irradiated lymphocytes is less than in unirradiated cultures. At 30 hr, the incorporation of thymidine in lymphocytes irradiated with 400 and 800 rads was 23 and 9% of controls, respectively. Similar results were obtained in 4 different experiments. This difference was not observed in our earlier studies (1), presumably because the cultures were not harvested at frequent enough intervals. After this lag, the increase in thymidine incorporation in irradiated lymphocytes parallels that in unirradiated controls until 40 hr after PHA stimulation. Thereafter, thymidine incorporation in control cultures continues to increase, while in irradiated lymphocytes the rate of DNA synthesis declines. In these partially synchronous cultures, the time of this decline corresponds to at least the second round of DNA replication (34), suggesting irradiated lymphocytes fail to undergo a second round of DNA replication.

In contrast to the effect of X-irradiation on thymidine incorporation, the timing for the induction of DNA polymerase and stimulation in uridine and amino acid incorporation was similar in irradiated and unirradiated lymphocytes (Chart 5). However, the magnitude of increase of each parameter was significantly reduced in irradiated cells. As observed in Chart 4 (82 hr after PHA stimulation), the inhibition in DNA polymerase was relatively less than that of RNA and protein synthesis. It should be noted that the decline in DNA polymerase after 64 hr in irradiated lymphocytes was relatively less than in controls. This dual response, early inhibition followed by less rapid decline, results in the apparent lack of effect of X-irradiation on DNA polymerase activity in cultures harvested at later periods (see Charts 1 and 3).

Morphological Studies. From the data presented, it can be surmised that DNA synthesis in irradiated lymphocytes is only slightly delayed but then proceeds normally during the first round of cell division. This conclusion is in accord with the similar morphological changes observed in irradiated and unirradiated lymphocytes (Chart 6). Between 24 and 40 hr after the addition of PHA, the percentage of small lymphocytes decreases and the percentage of labeled blasts increases. There is no significant difference in the percentage of these cells in lymphocyte cultures irradiated with 400 rads and in control cultures. However, at 92, 116, and 140 hr after PHA, the percentage of labeled lymphoblasts in irradiated cultures is lower than in controls. Conversely, the percentage of nondividing small lymphocytes is greater in the irradiated cultures.

Cell Cycle Studies by Cytofluorometry. The relative distribution of cells through different stages of cell cycle at 46 and 62 hr after the addition of PHA to irradiated and unirradiated lymphocytes was studied by cytofluorometry (Chart 7). In accord with the morphological studies presented above, at 46 hr

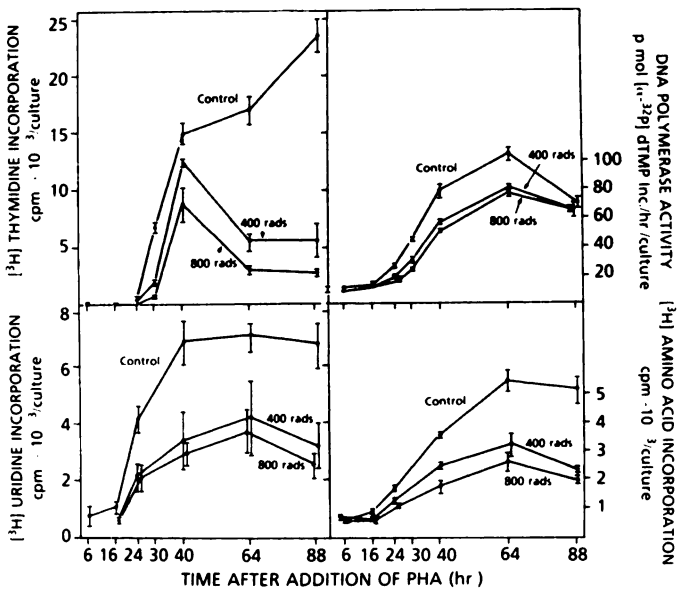

Chart 5. Time course of metabolic changes after PHA stimulation. Three sets of irradiated lymphocyte cultures, along with unirradiated controls, were harvested at 6, 16, 24, 30, 40, 64, and 88 hr after the addition of PHA. In one set, thymidine incorporation (*left upper*) and DNA polymerase activity (*right upper*), in another set, uridine incorporation (*left lower*), and in the third set, amino acid incorporation (*right lower*) was measured. *Point*, mean of triplicate cultures; *bars*, S.D.

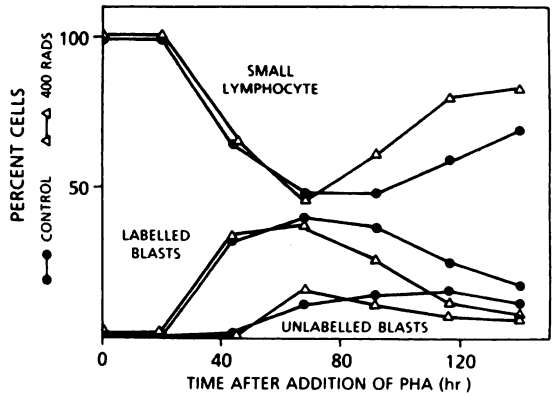

Chart 6. Morphological changes. At different times after the addition of PHA, unirradiated and irradiated lymphocytes were pulse labeled with [^{3}H]thymidine. Radioautographs were developed as described in "Materials and Methods," and afterwards the slides were stained with Giemsa stain. A total of 1000 cells from each slide were studied and classified into small lymphocytes, labeled blasts, and unlabeled blasts. The results are given as the percentage of each type.

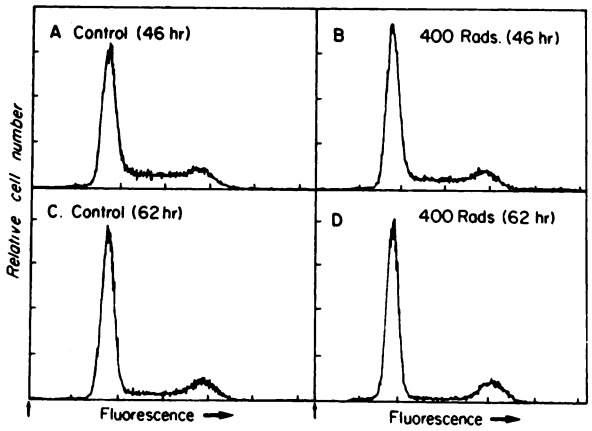

Chart 7. Analysis of cell cycle. Cytofluorometric tracings of PHA-stimulated lymphocytes were obtained as given in "Materials and Methods." In *A* and *B*, cultures were harvested at 46 hr. In *C* and *D*, cultures were harvested at 62 hr. *A* and *C* are unirradiated controls; *B* and *D* are lymphocytes exposed to 400 rads. The first peak corresponds to G_1, the plateau to S phase, and the second peak to G_2 and M.

after PHA stimulation, there was no significant difference in the percentage of cells at different stages of cell cycle in irradiated and control cells (Table 2). However, at 62 hr after PHA, there was a dose-dependent accumulation of cells in G_2 + M stage of cell cycle, as well as in G_1. There was a corresponding decrease in S phase cells. It is concluded that, in irradiated lymphocytes, the passage of cells from G_2, M, and G_1 stages to S phase is blocked during second and subsequent rounds of the cell cycle.

DISCUSSION

The results reported in this paper show that X-irradiation of human lymphocytes delays the onset of DNA synthesis after PHA stimulation. This initial delay in DNA synthesis may correspond to the prolongation of G_1 phase that is observed after X-irradiation of either continuously dividing or slowly dividing cell populations (32). However, by 44 hr in culture, thymidine incorporation in irradiated lymphocytes is nearly normal, indicating that, contrary to the suggestion of others (12), these cells do not undergo interphase death. After the first round of DNA replication, the irradiated lymphocytes fail to synthesize DNA in spite of normal amounts of DNA polymerase. This inhibition of DNA synthesis in irradiated lymphocytes at 60 hr is not due to cell death, since DNA polymerase activity continues to increase. This increase in polymerase activity has been shown to require continuous RNA and protein synthesis and, therefore, metabolically viable cells (2). Even though RNA synthesis is only partially decreased in irradiated lymphocytes, it is difficult to relate this change to the induction of specific proteins or to an RNA primer required for DNA replication, since X-irradiation has been reported to have different effects on the various species of RNA (14, 28). Cytofluorometric studies showed that at 62 hr there was a decrease in the percentage of cells in S phase. This is accompanied by an increase in the percentage of cells in G_2 + M and G_1 stages of the cell cycle. These combined results indicate that irradiated lymphocytes participate in the first round of DNA replication and do not enter into second or subsequent rounds. A reasonable explanation is that blockage of DNA synthesis is associated with unrepaired or misrepaired damage in the DNA template. Support for this hypothesis could be the demonstration of single-stranded DNA breaks on alkaline sucrose gradients.

Our findings suggest an explanation for the variations noted by cytogeneticists on the frequencies of chromosomal aberrations in X-irradiated human lymphocytes (5–7, 17, 31). When the X-irradiated leukocyte cultures were analyzed at 52 to 55 hr after stimulation with PHA, the frequency of 2-break aberrations was proportional to the dose of radiation. However, this frequency was at least 2-fold less when lymphocyte cultures were harvested at 72 hr or more after PHA stimulation. The data in this paper indicate that irradiated lymphocytes do not complete the second round of DNA synthesis and therefore stop proliferating. At later times, these cells may be diluted out by the continued proliferation of lymphocytes that have been less severely affected by radiation.

Inhibition of thymidine incorporation in irradiated lymphocytes upon PHA stimulation is probably not due to expansion of intracellular pools of TTP. Walters *et al.* (38) have shown that the size of nucleotide pools in Chinese hamster cells is not affected by X-irradiation. We have also found that addition of unlabeled thymidine to the culture medium causes a parallel

Table 2

Distribution of cells in different phases of the cell cycle as measured by cytofluorometry

Lymphocytes were irradiated with 0, 200, 400, and 800 rads in plastic tubes and stimulated with PHA. At 46 and 62 hr after the addition of PHA, the cells were collected by centrifugation and stained with propidium iodide in hypotonic citrate solution. Cytofluorometry was carried out as described by Krishan *et al.* (22). The percentages of cell cycle were calculated from the area under different parts of the cytofluorometric tracings.

	% of cells in			
	G_1	S	G_2 + M	S + (G_2 + M)
At 46 hr after PHA				
No radiation	71.2	17.9	10.9	28.8
200 rads	72.6	13.6	13.8	27.4
400 rads	70.3	14.8	14.9	29.7
800 rads	74.2	13.5	12.3	25.8
At 62 hr after PHA				
No radiation	60.9	23.8	15.3	39.1
200 rads	64.2	21.4	14.4	35.8
400 rads	67.4	16.1	16.4	32.5
800 rads	71.6	9.4	19.0	28.4

decrease in the incorporation of [^{3}H]thymidine in the irradiated as well as unirradiated lymphocytes. The latter indirectly implies that the pools of TTP precursors in irradiated cells are not altered in irradiated cells, as reported by Huang *et al.* (18).

In bacterial cells, DNA synthesis and the ability to form colonies has been shown to be 3 times more sensitive to X-irradiation than RNA and protein synthesis (33, 34). Also, the induction of enzymes in microorganisms is relatively radioresistant compared to the effect of X-rays on DNA replication (11). In contrast, enzyme induction in mammalian cells has been shown to be particularly sensitive to X-irradiation (3, 4, 15, 27, 37). In regenerating rat liver, irradiation of animals with 600 to 1000 rads before or within a few hr after partial hepatectomy has been reported to inhibit increases in deoxycytidine deaminase (30), thymidine kinase (37), and DNA polymerase activity (9). We find that irradiation of human lymphocytes with even up to 3200 rads only slightly diminished induction of DNA polymerase. Also, the induction of DNA polymerase was diminished less than the overall rate of RNA and protein synthesis.

Irradiation of lymphocytes with X-rays has been used to block the response in stimulator cells in one-way mixed lymphocyte culture reactions (16, 20, 26). This use of X-rays was based on the observation that X-irradiated lymphocytes do not incorporate thymidine upon PHA stimulation, yet they maintain their antigenicity and stimulate responder cells. However, our results indicate that X-irradiated lymphocytes continue to remain metabolically active, as is shown by the continued increase in DNA polymerase activity. They continue to synthesize RNA and protein and thus may synthesize and release mitogenic factors. This may account for the back-stimulation of cells in one-way mixed lymphocyte culture reactions where X-irradiation is used for inhibiting the response of the stimulating cells (35). As a practical point, much higher doses of irradiation (6400 rads or more) may be required to eliminate this back-stimulation.

REFERENCES

1. Agarwal, S. S., Brown, D. Q., Katz, E. J., and Loeb, L. A. Screening for deficits in DNA repair by the response of irradiated human lymphocytes to phytohemagglutinin. Cancer Res., *37:* 3594–3598, 1977.
2. Agarwal, S. S., and Loeb, L. A. Studies on the induction of DNA polymerase during transformation of human lymphocytes. Cancer Res., *32:* 107–133, 1972.
3. Beltz, R., Van Lancker, J., and Potter, V. R. X-Irradiation and nucleic acid synthesis. Cancer Res., *17:* 688–698, 1957.
4. Beltz, R. E. Comparison of the effects of X-radiation on the elevation of thymidine kinase and thymidylate synthetase during liver regeneration. Biochem. Biophys. Res. Commun., *9:* 78–83, 1962.
5. Bender, M. A. Human radiation cytogenetics. Adv. Radiat. Biol., *3:* 215–273, 1969.
6. Bender, M. A., and Brewen, J. G. Factors influencing chromosome aberration yields in the human peripheral leukocyte system. Mutat. Res., *8:* 383–399, 1969.
7. Bender, M. A., and Gooch, P. C. Types and rates of X-ray induced chromosome aberrations in human blood irradiated *in vitro*. Proc. Natl. Acad. Sci. U. S. A., *48:* 522–532, 1962.
8. Bertazzoni, U., Stefanini, M., Noy, G. P., Giulotto, E., Nuzzo, F., Falaschi, A., and Spadari, S. Variations of DNA polymerases-α and -β during prolonged stimulation of human lymphocytes. Proc. Natl. Acad. Sci. U. S. A., *73:* 785–789, 1976.
9. Bollum, F. J., Andergg, J. W., and McElya, A. B. Nucleic acid metabolism in regenerating rat liver. VII. Effect of X-radiation on enzymes of DNA synthesis. Cancer Res., *20:* 138–143, 1960.
10. Boyum, A. Isolation of leukocytes from human blood. Scand. J. Clin. Lab. Invest., *21:* 31–50, 1968.
11. Dewey, D. L. X-Ray inactivation of inducible enzyme synthesis and the effect of oxygen and glycerol. Nature (Lond.), *194:* 158–160, 1962.
12. Dewey, W. C., and Brannon, R. B. X-Irradiation of equine peripheral blood lymphocytes stimulated with phytohemagglutinin *in vitro*. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med., *30:* 229–246, 1976.
13. Dube, D. K., Kunkel, T. A., Seal, G., and Loeb, L. A. Distinctive properties of mammalian DNA polymerases. Biochim. Biophys. Acta, *561:* 369–382, 1979.
14. Enger, M. D., Walter, R. A., and Campbell, E. W. Effects of ionizing radiation on RNA metabolism in cultured mammalian cells. Biochim. Biophys. Acta, *353:* 227–237, 1974.
15. Furuno, I., Iwasaki, T., and Matsudaira, H. Effect of X-irradiation on cell proliferation and DNA synthesis induced by administration of isoproteronol in salivary glands of the mouse. Radiat. Res., *57:* 431–441, 1974.
16. Harrison, M. R., and Paul, W. E. Stimulus-response in mixed lymphocyte reaction. J. Exp. Med., *138:* 1602–1607, 1973.
17. Heddle, J. A., Evans, H. J., and Scott, D. Sampling time and the complexity of the human leukocyte culture system. *In:* H. J. Evans, W. M. Court Brown, and A. S. McLean (eds.), Human Radiation Cytogenetics, pp. 6–19. Amsterdam: North Holland Publishing Co., 1967.
18. Huang, A. T., Kremer, W. B., Laszlo, J., and Setlow, R. B. DNA repair in human leukemic lymphocytes. Nat. New Biol., *240:* 114–116, 1972.
19. Hungerford, D. A. Leukocytes cultured from small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. Stain Technol., *40:* 333–338, 1965.
20. Kasakura, S., and Lowenstein, L. Irradiated and preserved leukocytes in mixed leukocyte cultures. Proc. Soc. Exp. Biol. Med., *125:* 355–360, 1967.
21. Krishan, A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biol., *66:* 188–193, 1975.
22. Krishan, A., Pitman, A. W., Tattersal, M. H. N., Paika, K. D., Smith, D. C., and Frei, E. Flow microfluorometric patterns of human bone marrow and tumor cells in response to cancer chemotherapy. Cancer Res., *36:* 3813–3820, 1976.
23. Loeb, L. A. Molecular analysis of lymphocyte transformation. *In:* A. A. Gottlieb (ed.), Biology of Lymphoid Cells, pp. 103–132. New York: Academic Press, Inc., 1974.
24. Loeb, L. A., and Agarwal, S. S. DNA polymerase correlation with DNA replication during transformation of human lymphocytes. Exp. Cell Res., *66:* 299–304, 1971.
25. Loeb, L. A., Agarwal, S. S., and Woodside, A. M. Induction of DNA polymerase in human lymphocytes by phytohemagglutinin. Proc. Natl. Acad. Sci. U. S. A., *61:* 827–834, 1968.
26. Main, R. D., Cole, L. D., Jones, M. J., and Haire, H. M. DNA synthesis in mixed cultures of dog leukocytes: differential effect of X-irradiation and freeze thawing on cellular isoantigenicity. J. Immunol., *98:* 417–424, 1967.
27. Maine, R. K., Cole, L. J., and Walwick, E. R. X-Irradiation inhibition of induction of kidney enzymes catalysing synthesis of deoxyribonucleic acid *in vitro*. Nature (Lond.), *193:* 995–996, 1962.
28. Markov, G. G., Dessev, G. N., Russev, G. C., and Tsanev, R. G. Effects of γ-radiation on biosynthesis of different types of ribonucleic acids in normal and regenerating liver. Biochem. J., *146:* 41–51, 1975.
29. Mayer, R. J., Smith, R. G., and Gallo, R. C. DNA-metabolizing enzyme in normal human lymphoid cells. VI. Induction of DNA polymerases-α, -β, and -γ following stimulation with phytohemagglutinin. Blood, *46:* 509–518, 1975.
30. Myers, D. K., Hemphill, C. A., and Townsend, C. M. Deoxycytidylate deaminase levels in regenerating rat liver. Can. J. Biochem. Physiol., *39:* 1043–1054, 1961.
31. Norman, A., Ottoman, R. E., Sasaki, M., and Veomett, R. C. The frequency of dicentrics in human leukocytes irradiated *in vivo* and *in vitro*. Radiology, *83:* 108–110, 1964.
32. Okada, S. Radiation Biochemistry, Vol. 1, p. 190. New York: Academic Press, Inc., 1969.
33. Pauly, H. Synthesis of protein, RNA, DNA and induced enzyme in *Bacterium cadaveris* after X-irradiation. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med., *6:* 221–231, 1963.
34. Pollard, E., and Vogler, C. Radiation action on some metabolic processes in *Escherichia coli*. Radiat. Res., *15:* 109–119, 1961.
35. Sasaki, M. S., and Norman, A. Proliferation of human lymphocytes in culture. Nature (Lond.), *210:* 913–914, 1966.
36. Thorsby, E. The human major histocompatibility system. Transplant. Rev., *18:* 51–129, 1974.
37. Van Lancker, J. L. Metabolic alterations after total body doses of X-irradiation. II. Incorporation of deoxycytidylic and thymidylic acid into purified DNA and nuclei in presence of regenerating-liver supernatant. Biochim. Biophys. Acta, *45:* 63–70, 1960.
38. Walters, R. A., Gurley, L. R., Tobey, R. A., Enger, M. D., and Ratliff, R. L. Effects of X-irradiation on DNA precursor metabolism and deoxyribonucleoside triphosphate pools in Chinese hamster cells. Radiat. Res., *60:* 173–201, 1974.