Human lymphocytes in heparinized blood were collected through a tetoron fiber column. About $5 \times 10^5$ lymphocytes in a milliliter of Eagle's essential medium containing 20% autoserum were cultured with phytohemagglutinin (PHA) and exposed to x-irradiation. The results were as follows: (1) Morphological blast formation was not affected by x-rays below 300 R. About 60% of lymphocytes could transform even after an exposure to 2,000 R. (2) Autoradiography after continuous labeling with $^3$H-thymidine proved that entering of the lymphocytes into the DNA synthetic phase was suppressed by radiation. The percentage of cells entering the first DNA synthesis was dependent on the radiation dose; 96, 92, 73, 48, 37, 42, and 36% after the irradiation of 0, 150, 300, 500, 1,000, 1,500, and 2,000 R, respectively, 2 to 3 hours after the addition of PHA. (3) Accumulation of mitosis by colchicine was prevented by irradiation depending on doses; 72, 36, 37, 8, 8, and 5% of original lymphocytes entered the first mitosis after the irradiation of 0, 140, 300, 500, 1,000 and 2,000 R, respectively, at 2 to 3 hours culture. At about 2,000 R, not more than 10% of stimulated lymphocytes entered mitosis in 5 days' incubation.

Phytohemagglutinin (PHA) causes an abrupt increase in RNA synthesis, a progressive cell enlargement, DNA synthesis and cell division in human lymphocytes. In a study of this system, Schrek and Stefani found that PHA protected lymphocytes from cytocidal effect of radiation. This finding casts a light on the mechanism of radiation effects on transforming cells. Small lymphocytes, resting and non-dividing, are destroyed by radiation in a short time. This type of cell destruction, interphase death, is thought to be different from reproductive death of proliferating cells. In this experiment we have investigated what process in the transition of small lymphocytes into proliferating cells is affected by x-ray of various doses.

**Materials and Methods**

*Preparation of lymphocyte suspension.* Ten to 100 ml of venous blood were obtained from healthy male volunteers of 21 to 25 years of age. They had been submitted to a preliminary examination for the transformation index of lymphocytes, and only those subjects had been selected for the present study who had shown the indexes higher than 90%. Heparinized blood was allowed to stand with 0.6% dextran (mol. wt 150,000) for one hour at room temperature for the sedimentation of blood cells. The upper leukocyte-rich layer was transferred onto a tetoron fiber column (Toray Industries, Inc.) to remove...
granulocytes and washed with Eagle's minimum essential medium (MEM). Final cell suspension was prepared to give a lymphocyte concentration of about $5 \times 10^5$ cells in $1 \text{ ml}$ of MEM containing 20% autoserum. Usually 90 to 99% of nucleated cells in the suspension were lymphocytes. Phytohemagglutinin-M (Difco) was added to a final concentration of 2 $\mu$g in a milliliter of cell suspension.

**Differential cell count.** One milliliter of the cell suspension was transferred into a test tube and incubated for 10 minutes with 0.05 ml of 1% Triton-X-100 to separate aggregated cells. Cell count was made on a hemocytometer after the suspension was mixed with 1 ml of Türk's solution.

**Autoradiography.** Two microcuries of tritiated thymidine were added to the 30 hours culture with PHA, and smears were made after continuous labeling for 30 hours. Smears were air-dried, fixed with methanol, washed with 5% perchloric acid to remove free thymidine and then coated with Sakura NR–M2 dipping emulsion. After an exposure of 10 days, smears were stained with Giemsa and cells with more than 5 grains per cell were counted as 'labeled.'

**Irradiation.** The cell suspension in a culture bottle was irradiated with 180 kVp x-rays. The physical factors of irradiation were 20 mA, 0.5 mm Al+0.7 mm Cu filter added, half value layer of 1.22 mm Cu, 40 cm target-sample distance, and 65 R/min.

**RESULTS**

1) **Radiation effects on morphological changes of lymphocytes**

The normal lymphocyte in blood has a nucleus of 4 to 6 $\mu$ in diameter on the hemocytometer. After the addition of PHA, the lymphocyte enlarges with the synthesis of nucleic acid and protein, and becomes to acquire basophilic cytoplasm and prominent nucleolus. Large lymphoid cells of more than 7 $\mu$ in diameter on the hemocytometer were defined as PHA-reacted blast cells. Individual blast formation index was considerably different and the response of lymphocytes to PHA was statistically different from person to person. Therefore, blood had been obtained only from those persons whose lymphocytes demonstrated a blast formation index higher than 90%. Fig. 1 shows the dose-effect relationship on blast formation of lymphocytes from a donor. Irradiation less than 300 R at 2 hours' culture did not exert any effect on blast formation. About 60% of lymphocytes underwent transformation even after an exposure of 2,000 R, although the cell size was smaller in irradiated groups.

Figs. 2-a, 2-b, and 2-c indicate the difference in blast formation according to different intervals between the addition of PHA and irradiation. The cells were exposed to 300, 500 or 1,000 R at various culture time. As for 300 R, irradiation at any time after the addition of PHA demonstrated no effect on blast formation. Radiation before the addition of PHA had also no effect, provided that the intervals between the two treatments were less than 2 hours. In the case of 500 R, the transformation index after the irradiation at 0, 6, 10 or 20 hours' culture was not statistically different from the index of non-irradiated cells. With a dose of 1,000 R, lymphocytes of only two groups, irradiated at 10 and 20 hours' cultures, responded to PHA equally with non-irradiated lymphocytes. Radiation at other culture time depressed the response to PHA. These results
Fig. 1. Percentage of PHA-reacted blast cells at 72 hours' culture with PHA. Various doses were given 2 to 3 hours. The vertical line represents 95% confidential interval of each point.

indicate that irradiation at an earlier transformation stage was more effective in preventing blast formation, and that a large dose is required to block the blast formation in more advanced stages.

2) Radiation effects on stimulated lymphocytes entering the first DNA synthesis

The percentage of cells labeled on account of incorporated $^3$H-thymidine during the culture time of 30 to 60 hours on autoradiograms are plotted in Fig. 3.
Fig. 2. Relationship between blast formation rate at 72 hours of culture and the time of irradiation. Irradiation of 300 R (○) Fig. 2-a, 500 R (△) Fig. 2-b or 1,000 R (⋆) Fig. 2-c was given at various culture times with (+) or without (−) PHA. The shadowed area represents 95% confidential interval for the blast formation index of non-irradiated lymphocytes. The vertical line is 95% confidential interval of each percentage.

In the non-irradiated control, 96% of lymphocytes entered the first DNA-synthetic phase. In the irradiated groups, 92, 73, 48, 37, 42 and 36% of cells entered the S-phase after the irradiation of 150, 300, 500, 1,500 and 2,000 R, respectively. Cell suspension was exposed to x-rays at 2 to 3 hours of culture with PHA.
3) **Effects on mitosis**

One milliliter of cell suspension was sampled at various culture times and incubated with colchicine at a concentration of 4 μg/ml for several hours successively. After the incubation, the cells in metaphase were counted on a hemocytometer. Cumulative fraction of mitotic cells was expressed as the fraction of the original lymphocytes which entered the mitotic phase in a certain culture time with PHA. Cumulative fraction of mitotic cells \( (M_{t_i}) \) at the culture time \( t_i \) was calculated as follows:

\[
M_{t_i} = (1 + m_{t_1})(1 + m_{t_2}) \cdots (1 + m_{t_i}) - 1
\]

where \( m_{t_i} \) is the fraction of mitotic cells in the incubation time of \( t_i \) to \( t_i \). \( M_{t_i} \) also represents the increase in the lymphocyte number. The cumulative fraction of mitotic cells are shown in Fig. 4 for the different doses of irradiation. From the curve of OR, generation time was estimated at 21 to 25 hours, which were the time required for an increase of the mitotic fraction by 1. Sasaki and Norman \(^5\) reported the generation time of 22 hours by the method of chromosome labeling pattern. As indicated in Fig. 4, a longer delay of the first mitosis was observed after a larger dose. The ascending limb of the curve of accumulation of mitosis was also severely depressed by irradiation. The generation time of 22 hours being taken into consideration, the cumulative percentage of mitotic cells at a culture time of 22 hours from the onset of mitosis after mitotic delay may give a gross estimate of cells which entered the first mitosis. As indicated in Fig. 3, 72, 36, 37,
8, 8 and 5% of original lymphocytes entered the first mitosis after the irradiation of 0, 150, 300, 500, 1,000 and 2,000 R, respectively, at 2 to 3 hours of culture.

**Discussion**

Inhibitory effects of radiation are exhibited more clearly on mitosis than on morphological blast formation and DNA synthesis. Entering of the stimulated lymphocytes into the first mitosis was delayed and suppressed by irradiation. Such a marked block or long delay of mitosis after a moderate dose of less than 1,000 R has not been reported in other cultured cells. The division delay of 0.3 to 1.5 min/rad was reported with Hela cells and Chinese hamster cells.8–10

Our result is contradictory to that of Sasaki and Norman.5 They observed neither delay nor suppression of the cells in entering the mitosis after an irradiation of 500 R using colcemide at a concentration of 4 µg/ml. We tested both colchicine and colcemide at concentrations of 0.1 to 5 µg/ml and found that colcemide was much less effective in blocking stimulated lymphocytes in metaphase. The percentage of mitotic cells never exceeded 20% even after an incubation of 20
hours with colcemide, while the mitotic cells occupied 32% with colchicine for 9 hours at the same concentration of 4 µg/ml. The inhibitory effect of radiation on mitosis was possibly masked when colcemide was used. The difference of colchicine and colcemide, transformation index of lymphocytes, and the intervals between the addition of PHA and irradiation may be the cause of the disagreement in the results.

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References