Hum. Genet. 35, 57—70 (1976)

Human Genetics

The Human Leukocyte Test System

X. Higher Sensitivity to X-Irradiation in the G_0 Stage of the Cell Cycle of Early as Compared to Late Replicating Cells

Bernd Beek and Günter Obe

Institut für Genetik, Freie Universität Berlin, Arnimallee 5—7, 1000 Berlin 33

Summary. Leukocyte cultures were set up with X-irradiated whole blood (200 R). Cells starting with their DNA synthesis between 25 and 35 h after culture initiation ("early replicating cells") were pulse-labeled with tritiated thymidine ([^{3}H] TdR). Mitoses were collected with colcemid in adjacent intervals from 36 up to 72 h after culture initiation. At fixation times of 50, 56, 62, and 72 h enough mitoses for a determination of the frequencies of chromosomal aberrations (dicentric and ring chromosomes) were found. After that the preparations were processed for autoradiography. All mitoses analyzed for chromosomal aberrations were re-analyzed for labeling, and the frequencies of chromosomal aberrations in labeled (="early replicating cells") and unlabeled (="late replicating cells") mitoses were compared. At all fixation times, higher frequencies of dicentric chromosomes were found in labeled as compared to unlabeled mitoses, indicating a higher sensitivity of early replicating cells to X-irradiation in the G_0 stage of the cell cycle.

Introduction

There are many indications for the existence of at least two populations of cells in human leukocyte cultures after stimulation with phytohemagglutinin (PHA) (Jasińska et al., 1970; Johansen, 1972; Michalowski, 1963; Steffen and Stolzmann, 1969). In human leukocyte cultures set up with Ham's F-10 medium (Ham, 1963) and stimulated with PHA, a biphasic distribution of DNA synthesis maxima and the occurrence of two mitotic waves within the first 2 days after culture initiation has been described (Dudin et al., 1974). In additional experiments, cells replicating their DNA within the time span of the first DNA synthesis maximum were labeled by the incorporation of tritiated thymidine ([^{3}H] TdR), and all mitoses occurring up to 54 h after culture initiation were collected with colcemid. The results of the autoradiographic investigation of metaphases showed that within the population of first mitoses in this culture system, cells could be differentiated with respect to earlier or later occurrence of their

individual DNA synthesis period (Dudin et al., 1974). The biphasic distribution in the first G_1 phase of the cell cycle of the RNA synthesis pattern as well led us to assume the following sequence of events: In human leukocyte cultures set up with Ham's F-10 medium and stimulated with PHA, two subpopulations of cells possibly enter their autosynthetic cell cycle at different times, giving rise to two RNA and DNA synthesis maxima and in consequence to two mitotic waves within the first 2 days after culture initiation (Beek and Obe, 1975). This pattern, however, may well be altered by the use of different culture media and/or mitogens (Dudin et al., 1976; Obe et al., 1975 b; Obe et al., 1976).

Bender and Brewen (1969) postulated two subpopulations of cells in human leukocyte cultures, differing in their sensitivities to X-irradiation and in the time they reach mitosis. This has been concluded from the results of experiments in which leukocyte cultures, set up with TC medium 199 (Morgan et al., 1950) and with irradiated whole blood, were prepared at different times after culture initiation. In experiments with X-irradiated human leukocytes, synchronized by a G_1/S-block, Steffen and Michalowski (1973) compared the frequencies of asymmetric exchange aberrations (dicentric and ring chromosomes) in early and late dividing cells within the population of first mitoses after block release. In most experiments they found an increase of the aberration frequencies in later dividing cells. Since these authors could exclude in their experiments a significant selective mitotic delay of cells with aberrations, they discussed different sensitivities of subpopulations, ranging over a ratio of two, as a possible explanation for their results. In other series, however, they found the highest frequency of aberrations in early dividing cells. They concluded that possibly the quantities of early dividing cells with high chromosomal radiosensitivity may be extremely variable from one blood donor to another.

With Ham's F-10 cultures we found after a combined treatment with the bifunctional alkylating agent A 139 (2,5-bis-(methoxy-ethoxy)-3,6-bis-ethyleneimino-p-benzoquinone) and (^{3}H)TdR 3 times more chromatid interchanges in metaphases derived from early replicating cells (within 26—36 h after culture initiation), as compared to metaphases derived from late replicating cells (within 36—46 h after culture initiation). It has been confirmed by autoradiography that all metaphases analyzed in both series represented first mitoses after mutagen treatment (Beek and Obe, 1974).

In the present paper we want to show that early replicating cells in the Ham's F-10/PHA-system of human leukocyte cultures exhibit a higher sensitivity to X-irratiation in the G_0 stage of the cell cycle as compared to late replicating cells.

Materials and Methods

The standard leukocyte culture used in our laboratory contains 4.00 ml Ham's F-10 culture medium (Ham, 1963), 0.50 ml (about 10%) fetal bovine serum, 0.12 ml phytohemagglutinin (PHA) M (Difco), 0.02 ml of a penicillin-dihydrostreptomycin mixture (1.0 mg dihydrostreptomycin and 100 IU penicillin), and 0.40 ml venous blood.

For the following experiments 24 cultures were set up, each consisting of two complete standard cultures, with the use of disposable plastic centrifuge tubes as culture vessels. Before

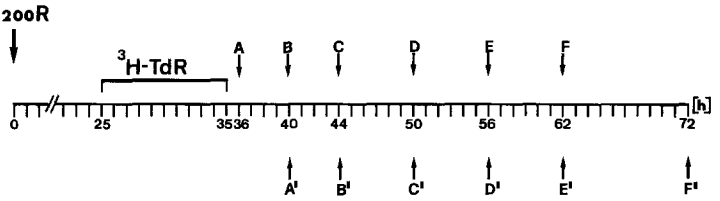

Fig. 1. Diagrammatic representation of experimental schedule. Irradiation of whole blood (G_0 stage of cell cycle) with 200 R X-rays, pulse-labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures. Addition of colcemid (*A* — *F*) to parallel cultures and preparations (*A'* — *F'*) at different times: *A*: addition of colcemid 36 h and preparation (A') 40 h, *B*: addition of colcemid 40 h and preparation (B') 44 h, *C*: addition of colcemid 44 h and preparation (C') 50 h, *D*: addition of colcemid 50 h and preparation (D') 56 h, *E*: addition of colcemid 56 h and preparation (E') 62 h, *F*: addition of colcemid 62 h and preparation (F') 72 h after culture initiation

addition to the cultures, freshly drawn whole blood of a normal healthy male donor was irradiated with 200 R X-rays (100 kV, 8 mA, 2.0 mm Al-filter, 58 cm focus distance, dose rate per min.: 30 R) at room temperature. Twelve of these cultures received 1 μCi/ml tritiated thymidine ([^{3}H]TdR); specific activity: 2 Ci/mM, Biochemical Center, Amersham) 25 h after culture initiation. The tracer was removed 35 h after culture initiation by washing 3 times with a prewarmed Hank's balanced salt solution containing "cold" thymidine (3×10^{-5}M) and centrifugation in a thermo-centrifuge at 37°C. After that the cell pellet was resuspended in conditioned medium from the remaining 12 "exchange" cultures.

In the pulse-labeled cultures mitoses were collected in adjacent intervals up to 72 h after culture initiation. For each of the following series two parallel cultures (I and II) were used. *A*: addition of colcemid 36 h, preparation 40 h after culture initiation (A'); *B*: addition of colcemid 40 h, preparation 44 h after culture initiation (B'); *C*: addition of colcemid 44 h, preparation 50 h after culture initiation (C'); *D*: addition of colcemid 50 h, preparation 56 h after culture initiation (D'); *E*: addition of colcemid 56 h, preparation 62 h after culture initiation (E'); *F*: addition of colcemid 62 h, preparation 72 h after culture initiation (F'). The steps of the experimental procedure are represented diagrammatically in Figure 1. Preparations were made as usual, including a hypotonic treatment with 0.075 M KCl for 7 min at room temperature, fixation with methanol : glacial acetic acid (3 : 1), and air drying. Preparations were stained with acetic orcein. In all series the frequencies of chromosomal aberrations (dicentric and ring chromosomes), as well as the frequencies of interphase nuclei with micronuclei and the percentages of mitoses were determined. After that the slides were processed for autoradiography with Kodak nuclear track emulsion NTB 3 and exposed for 3 weeks. After development of the autoradiographies with Kodak D-19 developer, as far as possible, all metaphases analyzed for chromosomal aberrations were re-analyzed for labeling. Mitoses with more than 10 silver grains over the chromosomes were counted as labeled.

Results

Frequencies of Mitoses and of Interphase Nuclei With Micronuclei

The results of the determination of the frequencies of mitoses and of interphase nuclei with micronuclei in all series are given in Table 1 and Figure 2. At the two earliest preparation times, 40 h (addition of colcemid 36 h) and 44 h (addition of colcemid 40 h) after culture initiation, no mitoses were found. After that the percentages of mitoses increased from a low level at 50 h (0.35% mean value;

Series	Mitoses (%)	Interphases with micronuclei (%)
40 h I	0.00	0.20
40 h II	0.00	0.00
44 h I	0.00	0.10
44 h II	0.00	0.30
50 h I	0.20	0.40
50 h II	0.50	0.50
56 h I	2.40	0.90
56 h II	1.40	0.90
62 h I	3.10	1.50
62 h II	3.20	1.90
72 h I	6.50	1.70
72 h II	5.80	3.00

Table 1. Percentages of mitoses and of interphase nuclei with micronuclei after irradiation of whole blood with 200 R X-rays, pulse-labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations of parallel cultures (I, II) at 40 h (addition of colcemid 36 h), 44 h (addition of colcemid 40 h), 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50 h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation. For each parallel series 1000 cells were analyzed

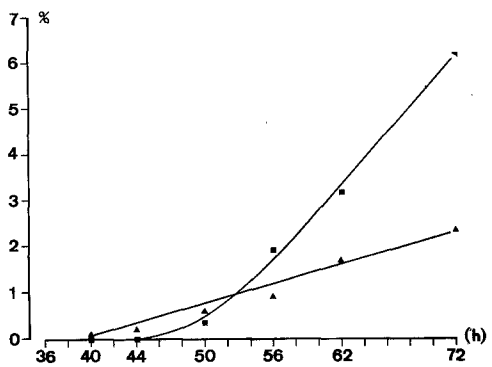

Fig. 2. Percentages of mitoses (■) and of interphase nuclei with micronuclei (▲) after irradiation of whole blood with 200 R X-rays, pulse-labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations at 40 h (addition of colcemid 36 h), 44 h (addition of colcemid 40 h), 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50 h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation. Pooled data from two parallel series (I and II, see Table 1). For each point given in figure, 2000 cells were analyzed

addition of colcemid 44 h) continously at 56 h (1.90% mean value; addition of colcemid 50 h) and 62 h (3.15% mean value; addition of colcemid 56 h) up to 72 h (6.15% mean value; addition of colcemid 62 h). As can be seen from Table 1, the results of the two parallel series (I and II) are similar.

The colcemid treatment is of the same duration (6 h) at the 50 h, 56 h, and 62 h preparation times. Thus, the increase of the frequencies of mitoses in these series reflects an increase of the mitotic activity with increasing culture time. At the 72 h preparation time the cultures were subjected to a longer colcemid treatment (10 h), so that the further increase of the percentages of mitoses in this series may in part be due to an accumulation of more mitoses.

As shown in Table 1 and Figure 2, the percentages of interphase nuclei with micronuclei increase continously with increasing culture time. The mean values of the percentages of cells with micronuclei are very low at the 40 h preparation time (0.10%), increasing at 44 h (0.20%), 50 h (0.45%), 56 h (0.90%), and 62 h (1.70%) up to 72 h (2.35%). As can be seen from Table 1, the results of the two parallel series (I and II) are similar.

Frequencies of Chromosomal Aberrations at the Different Preparation Times Before Autoradiography

The results of the determination of chromosomal aberrations (dicentric and ring chromosomes) at the different preparation times are presented in Table 2. In all series dicentric chromosomes and ring chromosomes as well were found to be associated with fragments. Fragments in cells without dicentric or ring chromosomes were not counted. The highest frequencies per cell of dicentric chromosomes and ring chromosomes as well were found at the earliest preparation time (50 h), when mitoses were present for analysis at all. At 56 h the lowest frequencies of both aberration types in all series occurred, increasing again at 62 h, and decreasing again in the 72 h series. Though the differences between the highest and lowest values of dicentric chromosomes (0.225 per cell at 50 h, 0.147 per cell at 56 h) and of ring chromosomes (0.050 per cell at 50 h, 0.030 per cell at 56 h) are not very high, the aberration frequencies seem to reflect a discontinous pattern.

In all series dicentric chromosomes were found to occur about 4 times more frequently than ring chromosomes.

Table 2. Frequencies of chromosomal aberrations (dicentric and ring chromosomes) after irradiation of whole blood with 200 R X-rays, pulse-labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations of parallel cultures (I, II) at 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50 h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation, and the number of mitoses analyzed

Series	Number of mitoses analyzed	Dicentric chromosomes			Ring chromosomes		
		Total	Per cell	% Mitoses	Total	Per cell	% Mitoses
50 h I	250	62	0.248	22.80	11	0.044	4.40
50 h II	88	14	0.159	13.64	6	0.068	6.82
50 h I + II	338	76	0.225	20.41	17	0.050	5.03
56 h I	300	43	0.143	14.00	9	0.030	3.00
56 h II	100	16	0.160	16.00	3	0.030	3.00
56 h I + II	400	59	0.147	14.50	12	0.030	3.00
62 h I	400	72	0.180	17.50	19	0.047	4.75
62 h II	300	56	0.187	17.67	12	0.040	4.00
62 h I + II	700	128	0.183	17.57	31	0.044	4.43
72 h I	270	40	0.148	13.33	13	0.048	4.81
72 h II	30	5	0.167	16.67	0	0.000	0.00
72 h I + II	300	45	0.150	13.33	13	0.043	4.33

The results of the two parallel series (I and II) are similar where high numbers of mitoses could be analyzed in both parallel series (56 h, 62 h). Differences between the two parallel series found at 50 h and 72 h may be an outcome of the low number of mitoses analyzed in one of the parallel series.

Frequencies of Labeled Mitoses at the Different Preparation Times

With few exceptions, all mitoses analyzed for chromosomal aberrations in the different series before autoradiography could be analyzed for labeling after autoradiography. The comparison of the numbers of mitoses analyzed in the different series before autoradiography (Table 2) with the numbers of labeled and unlabeled mitoses re-investigated (Table 3) shows that in series 50 h I three mitoses, in series 56 h I one mitosis, in series 62 h I five mitosis, and in series 72 h I two mitoses were lost. The aberration frequencies of the different series presented in Table 2 are not changed by the loss of these few mitoses.

The frequencies of labeled mitoses (more than 10 silver grains over the chromosomes) decline continously with increasing culture time (see Fig. 3). At the 50 h preparation time nearly all mitoses re-investigated after autoradiography (94.03%) were labeled and, at 56 h (82.96%) and 62 h (69.35%) the majority of cells were still labeled. At 72 h, labeling was found in 39.26% of the mitoses. As can be deduced from Figure 3, about 69 h after culture initiation equal frequencies of labeled and unlabeled mitoses are to be expected in these experiments.

Within the class of labeled mitoses a sub-class of "heavily labeled" mitoses with heavy labeling of all chromosomes could be distinguished. All heavily labeled mitoses exhibited a labeling pattern typically for first mitoses after pulse-labeling with (^{3}H)TdR, that is, both chromatids of the chromosomes were labeled at homologous sites.

As shown in Figure 3, the frequencies of heavily labeled mitoses decline more rapidly with increasing culture time than the frequencies of all labeled mitoses. From all mitoses analyzed after autoradiography 55.52% were heavily labeled at the 50 h preparation time, 34.09% at 56 h, 7.77% at 62 h, and 2.35% at 72 h. The total numbers of heavily labeled mitoses found in the different series are given in Table 4, except for series 72 h, where only seven heavily labeled mitoses were found in series 72 h I, and no heavily labeled mitoses at all in series 72 h II.

Frequencies of Chromosomal Aberrations in Labeled and Unlabeled Mitoses

The frequencies of dicentric chromosomes and ring chromosomes in labeled and unlabeled mitoses of all series are presented in Table 3. In unlabeled mitoses the frequencies of dicentric chromosomes were lower than in labeled mitoses at all preparation times. At the two earliest preparation times (50 h and 56 h), frequencies around 0.100 dicentric chromosomes per cell were found in unlabeled mitoses as compared to values of 0.235 per cell at 50 h and 0.157 per cell at 56 h in labeled mitoses. The lowest differences in the yields of dicentric chromosomes between labeled and unlabeled cells were found in the 62 h series. At 72 h again about twice as much dicentric chromosomes occurred in labeled as compared to

Table 3. Number of unlabeled and labeled mitoses after autoradiography, and frequencies of chromosomal aberrations (dicentric and ring chromosomes) in unlabeled and labeled mitoses. Irradiation of whole blood with 200 R X-rays, pulse labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations of parallel cultures (I, II) at 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50 h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation

Series	Unlabeled mitoses							Labeled mitoses						
	Number of mitoses	Dicentric chromosomes			Ring chromosomes			Number of mitoses	Dicentric chromosomes			Ring chromosomes		
		Total	Per cell	% mitoses	Total	Per cell	% mitoses		Total	Per cell	% mitoses	Total	Per cell	% mitoses
50 h I	17	2	0.118	11.76	1	0.059	5.89	230	60	0.261	23.91	10	0.043	4.35
50 h II	3	0	0.000	0.00	0	0.000	0.00	85	14	0.165	14.12	6	0.071	7.06
50 h I + II	20	2	0.100	10.00	1	0.050	5.00	315	74	0.235	21.27	16	0.051	5.10
56 h I	39	5	0.128	12.82	0	0.000	0.00	260	38	0.146	14.23	9	0.035	3.46
56 h II	29	2	0.069	6.90	0	0.000	0.00	71	14	0.197	19.72	3	0.042	4.23
56 h I + II	68	7	0.103	10.29	0	0.000	0.00	331	52	0.157	15.41	12	0.036	3.60
62 h I	132	24	0.182	16.67	8	0.061	6.06	263	45	0.171	16.73	10	0.038	3.80
62 h II	81	10	0.123	11.11	2	0.025	2.47	219	46	0.210	20.09	11	0.050	5.02
62 h I + II	213	34	0.160	14.55	10	0.047	4.69	482	91	0.189	18.26	21	0.044	4.36
72 h I	170	18	0.106	10.59	9	0.053	5.29	98	20	0.204	17.35	4	0.041	4.08
72 h II	11	2	0.182	18.18	0	0.000	0.00	19	3	0.158	15.79	0	0.000	0.00
72 h I + II	181	20	0.110	11.05	9	0.050	4.97	117	23	0.200	17.09	4	0.034	3.42

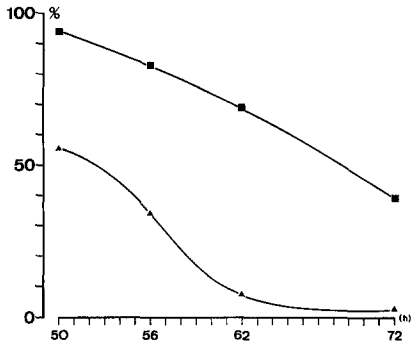

Fig. 3. Decline of labeled (■) and heavily labeled (▲) mitoses with increasing culture time. Percentages of labeled (more than 10 silver grains over the chromosomes) and heavily labeled (labeling over all chromosomes) mitoses after irradiation of whole blood with 200 R X-rays, pulse-labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations at 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50 h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation. Pooled data from two parallel series (I and II; for total numbers of labeled and heavily labeled mitoses see Tables 3 and 4). Heavily labeled mitoses, allowing investigation of the labeling patterns with respect to first and further mitoses after pulse-labeling, were definitely all first mitoses after incorporation of tracer

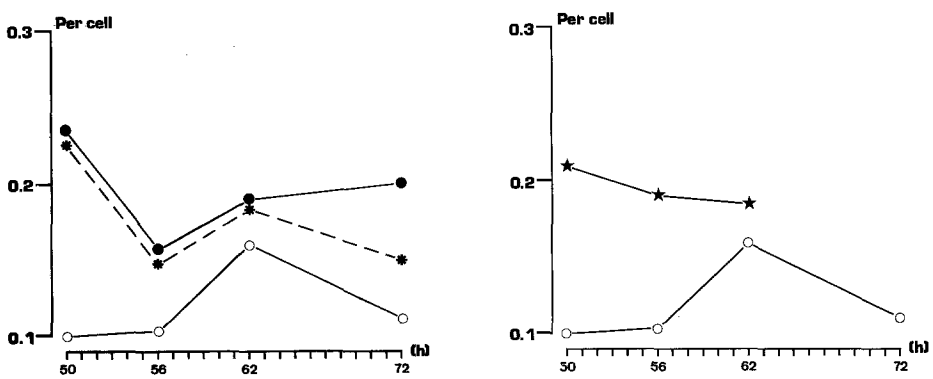

Fig. 4. Comparison of frequencies of dicentric chromosomes per cell in labeled (●) and unlabeled (○) mitoses at different culture times. Broken line represents the frequencies of dicentric chromosomes per cell at different culture times before autoradiography. Irradiation of whole blood with 200 R X-rays, pulse-labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations at 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation. Pooled data from two parallel series (I and II; see Tables 2 and 3)

Fig. 5. Comparison of frequencies of dicentric chromosomes per cell in heavily labeled (*) and unlabeled (○) mitoses after irradiation of whole blood with 200 R X-rays, pulse-labeling with (^{3}H)TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations at 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50 h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation. Pooled data from two parallel series (I and II; see Tables 3 and 4). All heavily labeled mitoses exhibited the labeling pattern typically for first mitoses after incorporation of tracer

unlabeled mitoses. Due to the predominance of labeled mitoses at the earlier preparation times, in the 50 h series only 20 unlabeled mitoses could be analyzed. The differences in the frequencies of dicentric chromosomes between labeled and unlabeled mitoses at the different preparation times are statistically significant, as indicated by the t-test for matched pair differences ($t=3.34$; df: 3; $P<0.05$).

Figure 4 shows the frequencies of dicentric chromosomes per cell in labeled and unlabeled mitoses, and the yield of dicentric chromosomes before autoradiography at the different preparation times (for a more detailed comparison, see Tables 2 and 3).

In contrast to the yields of dicentric chromosomes, the frequencies of ring chromosomes in labeled and unlabeled mitoses are within the same range in all series (about 0.040—0.050 per cell), with the exception of the 56 h preparation time where no ring chromosomes at all were found in 68 unlabeled mitoses analyzed. This may be due to the low frequency of ring chromosomes and the relatively small number of unlabeled mitoses in this series.

Within the sub-class of heavily labeled mitoses, all representing first mitoses after pulse-labeling with (^{3}H)TdR, a sufficient number of mitoses for a determination of aberration frequencies are only available in the 50 h, 56 h, and 62 h series (see Table 4). The frequencies of dicentric chromosomes in heavily labeled mitoses are within the range of those found in labeled mitoses (around 0.200 per cell), that is, at all preparation times they are also higher than in unlabeled ones (see Fig. 5). In contrast to the more irregular pattern of the yields of dicentric chromosomes at the different preparation times in the basic material (Table 2), and in labeled and unlabeled mitoses as well (Table 3), the frequencies of dicentric chromosomes per cell exhibit a slight but continuous decrease in heavily labeled mitoses with increasing culture time. The frequencies of ring chromosomes in heavily labeled mitoses are within the range found for all other series.

Table 4. Number of heavily labeled mitoses after autoradiography, and the frequencies of chromosomal aberrations (dicentric and ring chromosomes) in heavily labeled mitoses. Irradiation of whole blood with 200 R X-rays, pulse-labeling with (^{3}H) TdR from 25 h up to 35 h after initiation of human leukocyte cultures, and preparations of parallel cultures (I, II) at 50 h (addition of colcemid 44 h), 56 h (addition of colcemid 50 h), 62 h (addition of colcemid 56 h), and 72 h (addition of colcemid 62 h) after culture initiation

Series	Number of heavily labeled mitoses	Dicentric chromosomes			Ring chromosomes		
		Total	Per cell	% Mitoses	Total	Per cell	% Mitoses
50 h I	131	31	0.231	21.37	5	0.038	3.82
50 h II	55	8	0.145	14.55	3	0.055	5.45
50 h I + II	186	39	0.210	19.35	8	0.043	4.30
56 h I	120	25	0.208	20.00	3	0.025	2.50
56 h II	16	1	0.063	6.25	1	0.063	6.25
56 h I + II	136	26	0.191	18.38	4	0.029	2.94
62 h I	43	9	0.209	20.93	2	0.047	4.65
62 h II	11	1	0.091	9.09	0	0.000	0.00
62 h I + II	54	10	0.185	18.52	2	0.037	3.70

Table 5. Combined data from all series. Number of mitoses and frequencies of chromosomal aberrations (dicentric and ring chromosomes) per cell before autoradiography, and after autoradiography in unlabeled, labeled and heavily labeled mitoses.

		Number of mitoses	Dicentric chromosomes per cell	Ring chromosomes per cell
Mitoses before autoradiography		1738	0.176	0.042
Mitoses after autoradiography	Unlabeled mitoses	482	0.118	0.037
	Labeled mitoses	1245	0.195	0.041
	Heavily labeled mitoses	383	0.196	0.037

Table 5 shows the pooled data from all preparation times and presents a comparison of the frequencies of dicentric chromosomes and ring chromosomes per cell in all mitoses analyzed before autoradiography, in all unlabeled, labeled, and heavily labeled mitoses. As shown in the table, the frequencies of dicentric chromosomes in labeled and heavily labeled mitoses from all series are higher than in unlabeled mitoses. The frequencies of ring chromosomes are within the same range in all classes. The difference between the yield of dicentric chromosomes in labeled and unlabeled mitoses pooled from all preparation times is statistically significant, as indicated by the χ^2-test ($\chi^2 = 14.32$; df: 1; $P < 0.001$).

Discussion

In human leukocyte cultures set up with Ham's F-10 medium and stimulated with PHA, cells could be distinguished with respect to earlier or later occurrence of their DNA synthesis period and the time they reach mitoses (see *Introduction*). Thus, the basic idea for the experimental schedule used in the present investigation was to combine X-irradiation of unstimulated cells in the G_0 stage of the cell cycle with a differentiation of early and late replicating cells. Cells at least starting to replicate their DNA within the first 10 h of DNA synthesis (25—35 h) detectable in this culture system (Dudin et al., 1974) were pulse-labeled with (^{3}H)TdR and later identified as early replicating cells by autoradiography. Unlabeled mitoses, in turn, must be derived from cells entering the DNA synthesis period after 35 h in culture.

As may be seen from Table 3 and Figure 4, at all fixation times higher yields of dicentric chromosomes were found in metaphases derived from early replicating as compared to late replicating cells. This indicates that the population of early replicating cells in human leukocyte cultures exhibits a higher sensitivity to X-irradiation in the G_0 stage of the cell cycle.

In contrast to the yields of dicentric chromosomes, the frequencies of ring chromosomes were within the same range in labeled and unlabeled mitoses (Table 3). Due to the low frequency of ring chromosomes in all series, a definite conclusion is not possible.

Variations in the frequencies of radiation-induced chromosomal aberrations at different fixation times in human leukocyte cultures may be an outcome of mitotic selection of cells with aberrations (Buckton and Pike, 1964). For an exact determination of aberration frequencies it is therefore necessary to analyze only first mitoses after aberration induction. The heterogeneous aberration yields found at different fixation times after irradiation of human leukocyte cultures set up with TC medium 199 have been attributed only to "the appearance of the second and subsequent mitoses in culture which have an enhanced proportion of undamaged cells" (Heddle et al., 1967). It is therefore necessary to discuss whether the results of our present investigation may be influenced by the occurrence of second or further mitoses after aberration induction. First mitoses occur in the Ham's F-10/PHA-system in non-irradiated cultures around 35 h after culture initiation (Dudin et al., 1974). To analyze all mitoses with respect to chromosomal aberrations and labeling from the earliest occurrence up to 72 h after culture start, in the present investigation mitoses were collected by colcemid in adjacent intervals from 36—72 h after culture initiation (see Fig. 1). As may be seen from the determination of the frequencies of mitoses (Table 1, Fig. 2), in the present experimental series first low amounts of mitoses occurred within 44 h and 50 h after culture initiation, indicating a cell cycle delay of at least 9 h. This may be induced by the X-irradiation and by the further experimental procedure (i.e., pulse-labeling, washing and centrifugation, medium exchange). A mitotic delay induced by (^{3}H)TdR-labeling has been assumed by Bender and Brewen (1969). Up to 62 h after culture initiation the majority of metaphases were labeled, i.e., derived from early replicating cells. About 69 h after culture start equal amounts of metaphases from early and late replicating cells would be expected in the present experiments (see Fig. 3). This is in contrast to labeling experiments in non-irradiated leukocyte cultures of the same system (Dudin et al., 1974). Equal amounts of labeled and unlabeled metaphases were found in these experiments after pulse-labeling with (^{3}H)TdR from 26—36 h and colcemid treatment from 38—54 h after culture initiation. Though the effects of the different colcemid treatments have to be considered, this may indicate a cell cycle delay, possibly ranging between 10 and 15 h in our present experiments. In bromodeoxyuridine (BUdR) labeling experiments with the Ham's F-10/PHA-system we found at 72 h culture time (including a 4½ h colcemid treatment) in non-irradiated cultures the majority of mitoses undergoing the second division after culture initiation (Obe and Beek, 1975). About 57% second mitoses, 23% third and 20% first mitoses were found. The presence of first, second and third mitoses as well indicates that after a culture time of 3 days the initial relative synchrony of this culture system has been lost. Considering a cell cycle delay of about 10—15 h in the present investigation, a change of the pattern of mitoses toward a predominance of first mitoses still at 72 h after culture initiation seems to be possible. This is supported by the finding that in all series dicentric and ring chromosomes were found to be associated with fragments, and further by the absence of polyploid metaphases and metaphases with micronucleus-derived premature chromosome condensations (PCC), though micronuclei occur in increasing amounts (see Fig. 2). Exchange-type aberrations without fragments, polyploid mitoses (see Bender and Brewen, 1969; Heddle et al., 1967), and mitoses with micronucleus-derived PCC (see Obe et al., 1975a, b; Obe

and Beek, 1975; Dudin et al., 1976; Obe et al., 1976) have been used as indicators for at least second mitoses after the induction of chromosomal aberrations in different culture systems.

A predominance of first mitoses up to 72 h in our experimental series is indicated even more conclusive by the distribution of labeled cells. As already stated, in the present experiment the *early replicating,* which are also the *early dividing* cells, were labeled. As late as at 72 h after culture initiation more than 30% of the metaphases (see Fig. 3) are still derived from early replicating cells. All these labeled mitoses might be first mitoses after culture initiation. This is indicated by the finding that all mitoses allowing an exact determination of the labeling pattern with respect to the segregation of newly synthesized DNA (Taylor et al., 1957), the "sub-class" of heavily labeled mitoses, were labeled at homologous sites of both chromatids. At the earliest fixation time (50 h) more than 50% of all metaphases analyzed were heavily labeled (definitely first mitoses after pulse-labeling) (see Fig. 3). Thus, if second mitoses would occur at all, one should expect to find at least some metaphases with the labeling pattern typical for second mitoses. This, however, has not been the case, even at the latest fixation time (72 h). Furthermore, if it is improbable that within the population of early dividing cells second or subsequent mitoses occur in our series, the presence of second mitoses in unlabeled (*late dividing*) cells is even more improbable.

If nevertheless second mitoses would occur in our series, the results could be influenced only towards a reduction of the differences between the aberration yields in labeled and unlabeled mitoses; second mitoses are likely to occur at first in the population of early dividing cells with the higher aberration yield. Thus, a mitotic selection of aberration-bearing cells would affect mainly the early dividing cells, leading to a lower aberration yield in labeled metaphases. This may possibly be proved by a comparison of the aberration yields in heavily labeled mitoses (definitely first mitoses of early replicating cells) and mitoses labeled at all. If mitotic selection would affect the population of labeled mitoses, the aberration frequencies should be considerably higher in the sub-class of heavily labeled mitoses as compared to the labeled mitoses as a whole. As can be seen in Figures 4 and 5 and Table 5, the frequencies of dicentric chromosomes in heavily labeled mitoses are similar to the frequencies of dicentric chromosomes in mitoses labeled at all. This indicates that at least no considerable mitotic selection of aberration-bearing cells occurs in the population of early dividing cells. Therefore, a mitotic selection in late dividing cells is even more improbable.

It is well known that the incorporation of (^{3}H)TdR may produce chromosome aberrations (Bender et al., 1962; Brewen and Olivieri, 1966; Cleaver and Thomas, 1972; Dewey et al., 1965). Aberrations induced by the tracer would occur in the first post-labeling mitoses as *chromatid damage.* Asymmetric chromatid translocations may appear as dicentric chromosomes in the second post-labeling mitoses ("derived aberrations"). As already stated, the presence of second mitoses after culture initiation is very unlikely in our series. Moreover, since even in heavily labeled mitoses no chromatid translocations were found, an influence of the incorporation of (^{3}H)TdR on our results may be excluded.

Considering all the above-mentioned possibilities we feel that the results of our experiments reflect a true difference in the sensitivity to X-irradiation in the G_0 stage of the cell cycle of early as compared to late replicating cells. It is highly speculative to give reasons for this. Some differences in the physiological state of G_0 stage cells may be responsible for different radiosensitivities (repair capacities?) and different progression through the cell cycle as well. Especially in view of possible variations of the quantities of early dividing cells from one blood donor to another assumed by Steffen and Michalowski (1973), different sensitivities of subpopulations in human leukocyte cultures either to X-irradiation or to chemical mutagens (Beek and Obe, 1974) may account for a variety of uncertain or conflicting results.

References

Beek, B., Obe, G.: The human leukocyte test system II. Different sensitivities of sub-populations to a chemical mutagen. Mutat. Res. **24**, 395—398 (1974)

Beek, B., Obe, G.: The human leukocyte test system IV. The RNA-synthesis pattern in the first G_1-phase of the cell cycle after stimulation with PHA. Mutat. Res. **29**, 165—168 (1975)

Bender, M. A., Gooch, P. C., Prescott, D. M.: Aberrations induced in human leukocyte chromosomes by (^{3}H)labeled nucleosides. Cytogenetics **1**, 65—74 (1962)

Bender, M . A., Brewen, J. G.: Factors influencing chromosome aberration yields in the human peripheral leukocyte system. Mutat. Res. **8**, 383—399 (1969)

Brewen, J. G., Olivieri, G.:The kinetics of chromatid aberrations induced in Chinese hamster cells by tritium-labeled thymidine. Radiat. Res. **28**, 779—792 (1966)

Buckton, K. E., Pike, M. C.: Chromosome investigations on lymphocytes from irradiated patients. Effect of time in culture. Nature (Lond.) **202**, 714—715 (1964)

Cleaver, J. E., Thomas, G. H.: Biological damage from intranuclear tritium: DNA strand breaks and their repair. Science **177**, 996—998 (1972)

Dewey, W. C., Humphrey, R. M., Jones, B. A.: Comparisons of tritiated thymidine, tritiated water, and cobalt-60 gamma rays in inducing chromosomal aberrations. Radiat. Res. **24**, 214—238 (1965)

Dudin, G., Beek, B., Obe, G.: The human leukocyte test system I. DNA synthesis and mitoses in PHA-stimulated 2-day cultures. Mutat. Res. **23**, 279—281 (1974)

Dudin, G., Beek, B., Obe, G.: The human leukocyte test system VIII. DNA synthesis and mitoses in 3-day cultures stimulated with pokeweed mitogen. Human Genet. **32**, 323—327 (1976)

Ham, R. G.: An improved nutrient solution for diploid Chinese hamster and human cell lines. Exp. Cell Res. **29**, 515—526 (1963)

Heddle, J. A., Evans, H. J., Scott, D.: Sampling time and the complexity of the human leukocyte culture system: In: Human Radiation Cytogenetics (eds. H. J. Evans, W. M. Court Brown, A. S. McLean), pp. 6—19. Amsterdam: North Holland 1967

Jasińska, J., Steffen, J. A., Michalowski, A.: Studies on *in vitro* lymphocyte proliferation in cultures synchronized by the inhibition of DNA synthesis II. Kinetics of the initiation of the proliferative response. Exp. Cell Res. **61**, 333—341 (1970)

Johansen, J.: DNA synthesis and distribution of first and second division in 72-h-cultures of human leukocytes. Experientia (Basel) **28**, 416—417 (1972)

Michalowski, A.: Time-course of DNA synthesis in human leukocyte cultures. Exp. Cell Res. **32**, 609—612 (1963)

Morgan, J. F., Morton, M. J., Parker, R. C.: Nutrition of animal cells in tissue culture. Initial studies on a synthetic medium. Proc. Soc. exp. Biol. (N.Y.) **73**, 1—8 (1950)

Obe, G., Beek, B., Vaidya, V. G.: The human leukocyte test system III. Premature chromosome condensation from chemically and X-ray induced micronuclei. Mutat. Res. **27**, 89—101 (1975a)

Obe, G., Beek, B., Dudin, G.: The human leukocyte test system V. DNA synthesis and mitoses in PHA-stimulated 3-day cultures. Humangenetik **28**, 295—302 (1975b)

Obe, G., Beek, B.: The human leukocyte test system VII. Further investigations concerning micronucleus-derived premature chromosome condensation. Humangenetik **30**, 143—154 (1975)

Obe, G., Brandt, K., Beek, B.: The human leukocyte test system IX. DNA synthesis and mitoses in PHA-stimulated 2-day cultures set up with Eagle's minimal essential medium (MEM). Human Genet. **33**, 263—268 (1976)

Steffen, J. A., Stolzmann, W. M.: Studies on *in vitro* lymphocyte proliferation in cultures synchronized by the inhibition of DNA synthesis I. Variability of S plus G_2 periods of first generation cells. Exp. Cell Res. **56**, 453—460 (1969)

Steffen, J. A., Michalowski, A.: Heterogeneous chromosomal radiosensitivity of phytohaemagglutinin-stimulated human blood lymphocytes in culture. Mutat. Res. **17**, 367—376 (1973)

Taylor, J. H., Woods, P. S., Hughes, W. L.: The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labelled thymidine. Proc. nat. Acad. Sci. (Wash.) **43**, 122—128 (1957)

Received July 7, 1976