The Action of Cytostatic Agents on the Chromosomes of Human Leukocytes in Vitro (Preliminary Communication)

By Klaus Erich Hampel, Bernard Kober, Dieter Rösch, Heinrich Gerhartz and Karl-Heinz Meinig

The Action of Cytostatic Agents on the Chromosomes of Human Leukocytes in Vitro (Preliminary Communication)

By KLAUS ERICH HAMPEL, BERNARD KOBER, DIETER RÖSCH, HEINRICH GERHARTZ AND KARL-HEINZ MEINIG

ALTHOUGH CYTOSTATIC AGENTS have been widely used in the treatment of malignant diseases for years, very little information has been available concerning the action of these compounds on human chromosomes.[1,5,6,8,11,12] It was therefore considered worthwhile to report preliminary quantitative data obtained from short-term cultures of human leukocytes.

MATERIALS AND METHODS

Four hundred and ten cultures of normal peripheral human leukocytes were prepared and treated according to the method of Moorhead et al.[16] with slight modifications. The blood was taken from 46 patients suffering from nonleukemic internal diseases, none of these having previously received cytostatic agents or irradiation. 0.02 ml. bactophytohemagglutinin M (Difco) were added to the heparinized blood samples, which were then kept for about 30 minutes at a temperature of 2–4 C. The culture medium (total amount 10 ml.) was 30 per cent autologous plasma, 70 per cent TC 199 (Difco). Penicillin, 0.26 mg./ml., and dihydrostreptomycin, 0.2 mg./ml., were added to suppress bacterial contaminants. The cultures originally containing 10^6 leukocytes/ml. medium were incubated for 72 hours at a temperature of 37.0 ± 0.2 C. Twenty-four hours before fixation, one of the water soluble cytostatic agents to be investigated was added to the cultures. Control cultures, which received only the solvent, were included in each series of experiments. At 4 hours before fixation, 0.004 mg. desacetylmethylcolchicin (colcemide) was added for each ml. of medium. Hypotonic treatment with distilled water 1:3 was carried out for exactly 10 minutes, followed by 5 minutes of centrifugation. The metaphases were spread by the air-drying technic of Rothfels and Siminovitch.[17] One hundred well-spread metaphases of each culture were evaluated. In 26 cultures (which had received maximum doses of antitumor drugs—i.e., doses which had chromosomal abnormalities in about 80–100 per cent of the metaphases) the mitotic rate was so depressed that only some 20 to 40 metaphases could be found suitable for analysis. The percentage of metaphases with chromosomal damage and the mean frequency of breaks per cell were counted. The mean frequency of breaks in the analyzable interchanges served as a measure for some metaphases which could not be accurately enumerated due to deformity. The number of such interchanges was multiplied with a factor, representing the average breakage frequency of the analyzable interchanges. The frequency of breaks in a few cells with almost completely destroyed chromosome structure after high doses was visually estimated. The chromosomes were grouped according to the Denver nomenclature.[4]

The following substances were used in these experiments: 2,4,6-triethyleneimino-1,3,5-triazin (triethylenemelamine, TEM, Hoechst), 2,3,5-tris-ethyleneimino-benzochinone (1.4)

From Medizinische Klinik der Freien Universitat Berlin, Division of Hematology, and Division of Nuclear Medicine, Berlin, Germany.

First submitted Jan. 11, 1965; accepted for publication May 6, 1965.

KLAUS ERICH HAMPEL, *Dr. med., Wissenschaftlicher Assistant.* BERNHARD KOBER, *cand. med.* DIETER RÖSCH, *cand. med. dent.* HEINRICH GERHARTZ, *Prof. Dr. med., Oberarzt.* KARL-HEINZ MENIG, *Diplom-Physiker.*

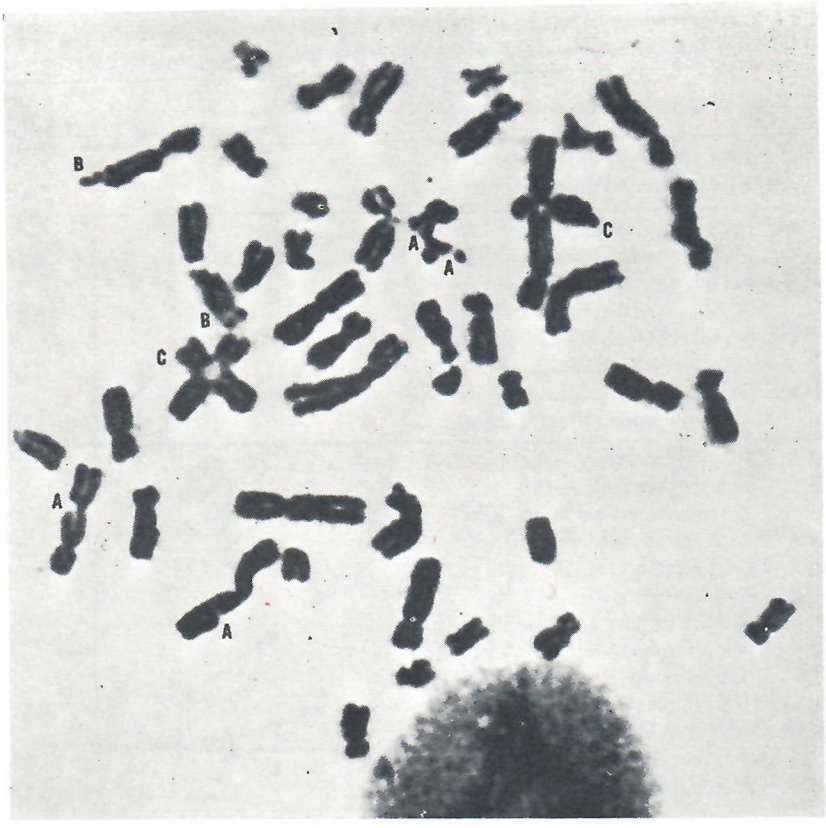

Fig. 1.—(a) Chromatid and (b) isochromatid breaks and two (c) interchanges in human chromosomes after treatment with 0.03 mg./ml. Thio-Tepa for 24 hours (154 B 6 K 20, 2000 ×).

(trenimon, Bayer), N,N′, N″-triethylenethiophosphoamide (thio-tepa, TESPA, Lederle), N,N-bis-(beta-chloroethyl)-N′, O-propylene phosphoric ester diamide (cyclophosphamide, endoxan, cytoxan, Asta), N, N-bis-(2-chloroethyl-) 0-(3-amino-propyl)-phosphoric acid amide ester (A 2, Asta), 4-amino-N^{10}-methyl-pteroylglutamic acid (amethopterin, methotrexate, Lederle), and 5-fluorodeoxyuridine (FUDR, Deutsche Hoffmann-La Roche).

Results and Discussion

The great majority of chromosome aberrations following cytostatic treatment of the leukocyte cultures consisted of chromatid and isochromatid breaks. Frequently minute fragments and chromatid interchanges were observed (fig. 1). After FUDR was added, however (6 and 24 hours before fixation), the formation of interchanges was very infrequent. This is in accordance with the results obtained by Taylor et al.[18] in *Vicia faba,* and Kihlman[13] as well.

Cyclophosphamide (0.2–0.4 mg./ml. culture medium) and amethopterin (0.1–100 μg./ml.) did not cause chromosomal damage when they were added directly to the cultures. However, 1 hour after the injection with cyclophosphamide, 500–1000 mg./Kg. body weight, plasma taken by heart puncture

Table 1.—*Chromosome Abnormalities in Cultured Human Leukocytes following Treatment for 24 Hours with Cyclophosphamide and its Activation Products*

	Percentage of Metaphases with Chromosomal Damage	Mean Breakage Frequency per Metaphase
Control plus 1 ml. plasma of untreated rat	6	0.06
Control plus 1 ml. plasma of untreated rat	5	0.06
(1) 0.2 mg. per ml. Cyclophosphamide directly added to the culture	13	0.14
(2) 0.2 mg. per ml. Cyclophosphamide directly added to the culture	5	0.07
(3) 0.4 mg. per ml. Cyclophosphamide directly added to the culture	3	0.03
(4) 0.4 mg. per ml. Cyclophosphamide directly added to the culture	4	0.04
(5) rat A: Cyclophosphamide, 500 mg. per Kg., intraperitoneally; 0.1 plasma added to the culture	26	0.32
(6) rat A: 0.2 ml. plasma added to the culture	34	0.50
(7) rat B: Cyclophosphamide, 1000 mg. per Kg., intraperitoneally; 0.2 ml. plasma added to the culture	29	0.69
(8) rat C: Cyclophosphamide, 500 mg. per Kg.. intravenously; 0.5 ml. plasma added to the culture	56	0.98
(9) rat D: Cyclophosphamide, 1000 mg. per Kg., intravenously; 0.5 ml. plasma added to the culture	87	2.58

Number of evaluated metaphases: 27 in culture 7; in all others, 100.

from rats, when added to the leukocyte cultures, resulted in numerous chromosomal aberrations similar to those caused by other alkylating agents (table 1). This is in accordance with the fact that cyclophosphamide (Cytoxan) is not active in vitro. Cytostatic effects are only demonstrable after activation of the compound in vivo, after perfusion of the isolated rat liver, after incubation with liver slices or microsome fractions of liver cells in presence of reduced triphosphopyridine nucleotid and oxygenium.[2] The active form of cyclophosphamide is present shortly after administration to mammals in the plasma and is excreted with the urine and bile.

No chromosomal damage was seen after methotrexate (fig. 2), even with extremely high dosage, nor after growth in folic acid free medium or after additional application of potassium cyanide, nor after passage through rats.

The quantitative analysis of chromosomal damage following cytostatic treatment of the leukocyte cultures is depicted in figure 2. All antitumor drugs used in our experiments except methotrexate showed an exponential dose dependent increase in the percentage of metaphases with chromosome aberrations. However, the mean count of breaks per metaphase rose approximately linearly with the applied dose (fig. 3). Due to considerable range of the re-

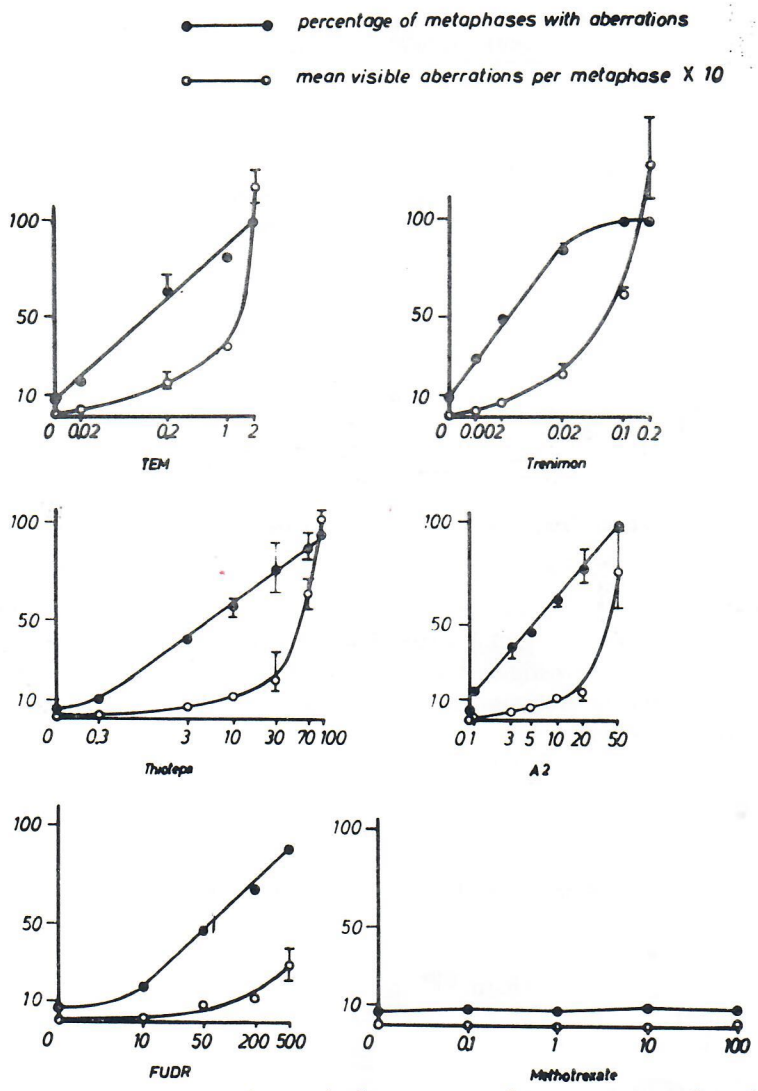

Fig. 2.—Quantitative analysis of chromosome aberrations in cultured human leukocytes caused by cytostatic agents.

sults with A 2, this line could not be fixed accurately. Similar results have been obtained in studies of plant material after x-irradiation.[14]

It is thus possible to establish a test for the cytogenetic action of cytostatic compounds on human chromosomes in vitro by taking as a standard the dose which causes chromosome breaks in 50 per cent of all metaphases in a culture. Another possibility is to calculate the theoretic dosage required to cause 5 breaks per metaphase. Table 2 shows these calculated doses in comparison with both the single doses and the remission doses clinically used in man as well as the DL^{1}_{50} of the rat. (The action of FUDR could not be exact-

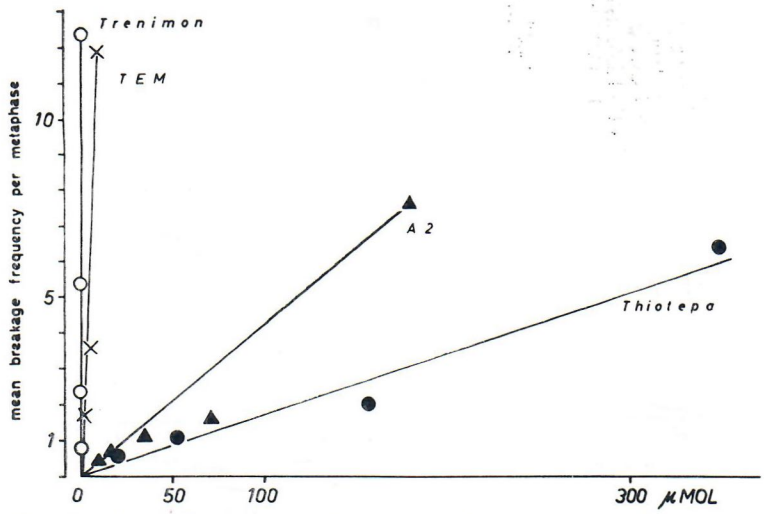

Fig. 3.—Breakage frequency of human chromosomes following cytostatic treatment.

ly compared with the other agents due to the presence of nucleotide precursors in the culture medium.) Table 2 shows, that the ratio of the cytostatic activity among the investigated antitumor drugs is approximately constant when administered in vivo and that the cytogenetic action on human chromosomes in vitro follows a similar pattern.

If an arbitrary value of 1000 units is used to represent the average measured total length of a female karyotype, the total length of a male karyotype (981 units) and the relative length of a single chromosome or chromosomal seg-

Table 2.—*Dosage Comparison of Cytostatic Agents*

	TEM	Trenimon	Thiotepa	A 2	FUDR
Single intravenous dose in man (mg.) (Gerhartz[9])	2–10	0.5–1.0	10	—	ca. 70
Intravenous remission dose in man (mg.) (Gerhartz[9])	35–70	5–7	180	—	?
DL^1_{50} in the rat (mg./Kg. i.p.) (Gerhartz,[10] Brock,[3] Roche[7])	1.1[10]	0.25[10]	8.4[10]	600[3]	350[7]
Dosage which causes chromosomal damage in 50 per cent of the metaphases in vitro (microgram per ml. culture medium)	0.1	0.045	5	5.5	60
Theoretical dosage required for 5 breaks per metaphase (micromole)	4	0.3	280	120	3000

Table 3.—*Localization of Breaks Caused by Cytostatic Agents in the Chromosomes of Human Leukocytes (Values/1000 Breaks)*

Chromosome No.	Arm	Segment	Random Distribution Male	Random Distribution Female	TEM f.	Trenimon m.	Thio-Tepa f.	A 2 m.
1	long	distal	15	15	45	49	73++	52
		intermed.	15	15	25	28	25	37++
		proximal	15	15	10	7	12	9
	short	d.	14	14	20	14	30+	15
		i.	14	14	10	11	8	16
		p.	14	14	2	6	6	6
2	long	d.	16	16	39	36	36	23
		i.	16	16	25	25	22	28
		p.	16	16	11	12	4	10
	short	d.	11	11	20	10	18	7
		i.	11	11	11	12	15	8
		p.	11	11	13	6	5	2
3	±	d.	23	23	28	23	21	28
		i.	23	23	8	17	14	20
		p.	23	23	8	2	2	2
4–5	long	d.	30	29	45	29	45	30
		i.	30	29	32	35	28	75++
		p.	30	29	8	11	9	6
	short	d.	11	11	12	11	12	8
		i.	11	11	8	8	8	12
		p.	11	11	2	5	2	2
6–12, X	long	d.	80	85	204	169	213	125
		i.	80	85	98	109	93	123+
		p.	80	85	46	48	30	37
	short	d.	40	43	49	72+	46	24+
		i.	40	43	31	23	28	37
		p.	40	43	18	18	14	11
13–15	long	d.	30	29	70+	42	59	54
		i.	30	29	35	63+	21	62+
		p.	30	29	11	8	6	12
	short		13	13	—	3	3	1
16	long	d.	6	6	4++	19	16	22
		i.	6	6	4	5	7	18++
		p.	6	6	3	3	1	7
	short		12	12	6	7	5	11
17–18	long	d.	14	14	10	15	15	12
		i.	14	14	9	4	6	15
		p.	14	14	3	2	—	4
	short		15	15	1	2	1	1
19–20	±	d.	15	15	9	13	9	3
		i.	15	15	2	1	10	7
		p.	15	15	—	1	—	1
21–22, Y	long	d.	11	8	1	11	17	8
		i.	11	8	1	2	2	4
		p.	11	8	1	3	3	7
	short		9	7	—	—	1	—
			997	1004	998	1000	1001	1002
Number of breaks counted					905	944	1028	1044

++: $\alpha \leq 0.05$ +: $\alpha \leq 0.10$

ment can be defined. The data of Levan and Nichols,[15] which are based on measurements of human chromosomes, were used. The values for a male karyotype were also expressed in parts of 1000 in table 3. If one assumed a random distribution of breaks in the chromosomes, the actually counted breaks in each chromosomal segment must be proportional to its relative length. If one assumed, furthermore, that one unit of chromosome length contained one break and that the total number of breaks are also 1000, the number of breaks in a chromosome segment must be equal to its relative length. The values given in table 3, columns 1 and 2, therefore represent not only the relative lengths of the chromosome segments in a male and female karyotype but also the number of breaks in these segments if a random distribution is assumed. For practical use it was thought best to follow Levan's proposal and to classify the human chromosomes as follows : Number 1, 2, 3, 4–5, 6–12, X, 13–15, 16, 17–18, 19–20, 21–22, Y.

The long and the short arms, where possible, were divided into 3 segments equal in size: *proximal, intermediate* and *distal* from the centromere.[12] If the values in table 3 in columns 1 and 2 are compared with the relative number of breaks found in the segments after the treatment of the cultures with cytostatic agents, it can be easily appreciated that the distribution of breaks followed a nonrandom pattern. An asymmetric distribution seems likely at least in some of the longer chromosomes. With great regularity the breakage frequency dropped from distal to proximal segments. Therefore the breaks in chromosomes 1 and 16 were not localized more often in the area of the secondary constrictions as recently reported for mitomycin C.[5] It seems also likely that different alkylating agents may lead to different distribution of breaks. When the x^2 test was applied, some of the differences in several segments proved to be significant. Further experiments demonstrating the reproducibility of these findings are necessary in the future. Although all the effect on human genetic material of antitumor therapy with cytostatic agents is not yet clarified, the chromosomal damage here seen in vitro would suggest similar effects in treated patients, especially in tissues with high mitotic activity.

Summary

Quantitative analysis of the chromosomal injuries following in vitro addition of various cytostatic agents to short-term human leukocyte cultures was carried out. The data obtained suggest that the cytogenic action of these various compounds in vitro follows a pattern similar to its action when administered therapeutically to man. The technics used may form the basis of further evaluation of the cytogenetic activity of these various compounds.

Summario in Interlingua

Esseva executate analyses quantitative del lesionage chromosomal post le addition in vitro de varie agentes cytostatic a culturas de breve duration de leucocytos human. Le datos obtenite suggere que la action cytogenic de iste varie compositos in vitro seque un configuration simile a illo de su action post administrationes therapeutic a humanos o experimental a rattos. Le technicas

describite va possibilemente formar le base de un evalutation additional del activitate cytogenetic de iste varie compositos.

REFERENCES

1. Arrighi, F. E., Hsu, T. C., and Bergsagel, D. E.: Chromosome damage in murine and human cells following cytoxan therapy. Texas Rep. Biol. Med. 20:545–549, 1962.
2. Brock, N.. and Hohorst, H.-J.: Über die Aktivierung von Cyclophosphamid in vivo und in vitro. Arzneimittelforsch. 13:1021–1031, 1963.
3. Brock, N.: Personal communication.
4. Denver study group: A proposed standard system of nomenclature of human mitotic chromosomes. J. A. M. A. 174:159–162, 1960.
5. Cohen, M. M., and Shaw, M. W.: Effects of mitomycin C on human chromosomes. J. Cell Biol. 23:386–395, 1964.
6. Conen, P. E., and Lansky, G. S.: Chromosome damage during nitrogen mustard therapy. Brit. Med. J. 2: 1055–1057, 1961.
7. Deutsche Hoffmann-La Roche: Personal communication.
8. Elves, M. W., Buttoo, A. S., Israels, M. C. G., and Wilkinson, J. F.: Chromosome changes caused by 6-azauridine during treatment of acute myeloblastic leukaemia. Brit. Med. J. 1: 156–159, 1963.
9. Gerhartz, H.: Chemotherapie der Hämoblastosen und bösartigen Geschwülste. Fortschr. Med. 81:607, 1963.
10. —: Vergleichende Untersuchungen zur Toxicität und Seitenwirkung moderner Cytostatica. Verh. Dtsch. Ges. inn. Med. 68:251–255, 1962.
11. Hampel, K. E., and Palme, G.: Chromosomenstudien bei akuter und chronischer myeloischer Leukämie. Neoplasma 11:113–122, 1964.
12. Hampel, K. E., and Gerhartz, H.: Strukturanomalien der Chromosomen menschlicher Leukozyten in vitro durch Triaethylenmelamin. Exp. Cell Res. 37:251–258, 1965.
13. Kihlman, B. A.: The production of chromatid aberrations by 5-fluorodeoxyuridine alone and in combination with X-rays and 8-ethoxy-caffeine. Caryologia 15:261–277, 1962.
14. Lea, D. E.: Actions of Radiations on Living Cells. New York, Cambridge University Press, 1955.
15. Levan, A., and Nichols, W. W.: Human chromosome lengths for use in distribution studies. Hereditas 51:378–380, 1964.
16. Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., and Hungerford, D. A.: Chromosome preparations of leukocytes cultured from human peripheral blood. Exp. Cell Res. 20:613–616, 1960.
17. Rothfels, K. H., and Siminovitch, L.: An air-drying technique for flattening chromosomes in mammalian cells grown in vitro. Stain Technol. 33:73–77, 1958.
18. Taylor, J. H., Haut, W. F., and Tung, J.: Effects of fluorodeoxyuridine on DNA replication, chromosome breakage and reunion. Proc. Nat. Acad. Sci. 48:190–198, 1962.