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ACTION OF cis-DICHLOROBIS (CYCLOPENTYLAMINE) PLATINUM(II) (cis-PAD) ON L5178Y CELLS OF TWO STRAINS INVERSELY CROSS-SENSITIVE TO X-RAYS AND UV-LIGHT

I. Cytotoxicity

I. Szumiel

Warszawa 1977

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DEPARTMENT OF RADIOBIOLOGY AND HEALTH PROTECTION

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I. CYTOTOXICITY

DZIAŁANIE cis-DWUCHLORO-BIS(CYKLOPENTYLOAMINO)PLATYNY(II)(cis-PAD) NA KOMÓRKI L5178Y DWÓCH SZCZEPÓW KRZYŻOWO WRAŻLIWYCH NA PROMIENIOWANIE X i ŚWIATŁO NADFIOLETOWE

I. CYTOTOKSYCZNOŚĆ

ДЕЙСТВИЕ цис-ДИХЛОРБИС(ЦИКЛОПЕНТИЛАМИН) ПЛАТИНЫ(II) (цис-ПАД) НА КЛЕТКИ L5178Y ДВУХ ШТАММОВ ПРОЯВЛЯЮЩИХ ПЕРЕКРЕСТНУЮ ЧУВСТВИТЕЛЬНОСТЬ К РЕНТГЕНОВСКИМ И УЛЬТРАФИОЛЕТОВЫМ ЛУЧАМ

I. ЦИТОТОКСИЧНОСТЬ

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Abstract

The response to cis-PAD, an antitumour platinum complex, was studied in two strains of murine lymphoma L5178Y cross-sensitive to X-rays and UV light. Dose-survival relationship, DNA synthesis, formation of chromatid aberrations, progression through the cell cycle, and growth and viability changes after 1 h cis-PAD treatment at 37°C were examined and compared with respective effects of X-rays and UV-light. In both strains studied, cis-PAD causes immediate inhibition of progression through the cell cycle, reduced rate of DNA synthesis, delayed appearance of chromatid aberrations and delayed death. However, there is a marked difference in sensitivity to cis-PAD between L5178Y-S strain (D_o ca. 5.8 μg/ml) and L5178Y-R strain (D_o ca. 2.5 μg/ml). In both strains a close resemblance was found between dose-survival relationship after cis-PAD and UV-light treatment, respectively.

Streszczenie

Zbadano działanie przeciwnowotworowego kompleksu platyny, cis-PAD, na komórki dwóch szczepów białaczki mysiej L5178Y, krzyżowo wrażliwych na promieniowanie X i światło nadfioletowe. Przedmiotem badań i porównania ze skutkami napromienienia promieniowaniem X i światłem UV były następujące skutki działania cis-PAD przez 1 godz. w temperaturze 37°C: spadek przeżywalności, hamowanie syntezy DNA, tworzenie aberacji chromatydowych, zaburzenia w przechodzeniu przez cykl komórkowy, zmiany wzrostu i żywotności. W obu badanych szczepach cis-PAD powoduje natychmiastowe zahamowanie przechodzenia przez cykl komórkowy, spowolnienie syntezy DNA oraz - po pewnej zwłoce - wytworzenie aberacji chromatydowych i śmierć komórek. Istotna różnica występuje natomiast we wrażliwości na cis-PAD; wartości D_o wynoszą ok. 5,8 μg/ml (L5178Y-S) i 2,5 μg/ml (L5178Y-R). Stwierdzono znaczne podobieństwo zależności dawka - przeżywalność obu szczepów po traktowaniu cis-PAD i ekspozycji na światło nadfioletowe.

Резюме

Исследовано действие противоопухолевого комплекса платины, цис-ПАД, на клетки двух штаммов мышиной лейкемии L5178Y, перекрестно чувствительных к рентгеновским и ультрафиолетовым лучам. Предметом исследований и сравнения с эффектами рентгеновского и ультрафиолетового облучения были следующие эффекты действия цис-ПАД в течение I часа в температуре 37°С: понижение выживаемости, торможение синтеза ДНК, индукция хроматидных аберраций, нарушение прогрессии через клеточный цикл, изменения жизнеспособности и роста. В обоих исследованных штаммах цис-ПАД вызывает непосредственно торможение движения клеток по клеточному циклу, замедление скорости синтеза ДНК и - спустя некоторое время - появление хроматидных аберраций и гибель клеток. Существенная разница была найдена в чувствительности к цис-ПАД: величины D_0 были 5,8 мкг/мл (L5178Y-S) и 2,5 мкг/мл (L5178Y-R). Было обнаружено заметное сходство зависимости доза-выживаемость обоих штаммов после действия цис-ПАД и экспозиции на ультрафиолетовые лучи.

CONTENTS

Abbreviations

CHO	- Chinese hamster ovary cells
cis-DDP	- cis-dichlorodiamineplatinum II
cis-PAD	- cis-dichlorobis(cyclopentylamine)platinum II
DMSO	- dimethylsulphoxide
dThd	- thymidine
ip	- intraperitoneal
LI	- labelling index
N_c	- number of cells in the control population
N_T	- number of cells in the treated population
N(M)	- fraction of mitotic cells
S_d	- sensitivity in terms of mitotic delay
T_D	- doubling time
T_G	- generation time
UV	- ultraviolet light.

1. INTRODUCTION

Discovery of the anti-tumour activity of platinum coordination complexes [1] prompted synthesis and testing of many compounds of this class [2-4]. Promising results obtained with a number of animal tumours [2-5] led to the hope that this class of compounds might be applied in human cancer therapy.

cis-Dichlorobis(cyclopentylamine)platinum(II) (cis-PAD), synthesized by Connors et al. [2,3] represents one of the most active platinum complexes of exceptionally high therapeutic index as determined by in vivo studies. The structure of cis-PAD is presented in Fig.1. Its action on Chinese hamster ovary (CHO) cells and its interaction with ionizing radiation were described previously [6-8]. A designation "DBCP" was used for the compound in that series of papers. Several months earlier the complex was designated PAD by the American authors [4]; therefore, abbreviation "cis-PAD" will be used in the present series of articles.

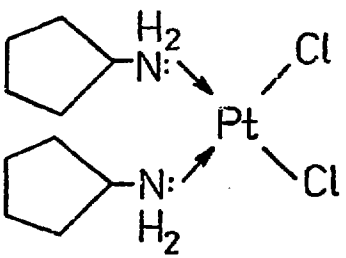


FIG.1. Structure of cis-PAD.

Numerous studies indicate [9-17] that both cis and trans platinum complexes react with DNA and that this is the major cause of their cytotoxicity; however, only cis complexes show anti-tumour activity in vivo. The reactions with DNA include intra- and inter-strand cross-link formation and mono-dentate and bidentate attack upon purine and pyrimidine bases. Until now, no individual type of lesion responsible for the anti-tumour effect of cis complexes has been identified.

It seems reasonable to assume that similar types of damage to target molecules induce similar phenomena at the cellular level. Therefore, comparison at the cellular level of effects caused by various DNA damaging agents may be informative when cellular systems are used, differing in susceptibility to these agents. A system particularly suitable for such comparison in vitro has been in use in this laboratory for some time. The system includes two closely related strains of L5178Y cells [18,19]: L5178Y-R, relatively resistant to X-rays and L5178Y-S, relatively sensitive to X-rays. Apart from the

difference in sensitivity to ionizing radiation, the two strains exhibit also a marked difference in sensitivity to UV-light. Interestingly, they are inversely cross-sensitive to these radiations [20, 21].

Effects of the cytotoxic action of cis-PAD on both L5178Y strains are described in this paper and compared - where possible - with the respective effects of X-rays and UV-light. These studies were aimed at a better understanding of the mechanisms underlying sensitivity to cis-PAD and recovery from damage inflicted by cis-PAD alone and in combination with radiation.

2. MATERIALS AND METHODS

2.1. Platinum complex

cis-PAD was kindly provided by Dr T.A.Connors and by Johnson Matthey and Co.Ltd. (London, UK).

2.2. Cell cultures

L5178Y cells were cultivated as suspensions in Fischer's medium [22] containing 8 % of calf serum (Państwowa Wytwórnia Surowic i Szczepionek, Lublin, Poland) and supplemented with 0.1 g/l neomycin.

The culture volume was ca 5 ml or ca 10 ml; the cultures were carried at 37°C in 10 ml or 25 ml conical flasks, respectively, stoppered with silicone rubber bungs or with ground glass stoppers lubricated with silicone grease. L5178Y-R cell population densities were maintained between 2.3×10^4 and 70×10^4 cells per millilitre, L5178Y-S cell population densities between 0.6×10^4 and 70×10^4 cells per millilitre, with suitable dilutions carried out every other day. The cultures growing at slower than the control rates after cis-PAD treatment were diluted at longer intervals. If in L5178Y cultures treated with cis-PAD cell population density had not increased for 4-5 days to a level making dilution possible, then the cultures were centrifuged for 7 min at ca 500 g and the supernatant replaced with a fresh portion of prewarmed Fischer's medium.

Cell population densities were determined using a Burker haemocytometer.

2.3. Treatment with cis-PAD

cis-PAD solution was prepared, at a concentration of 2 mg/ml in dimethylsulphoxide (DMSO, Reachim, USSR). The solution

obtained was immediately diluted 1:4 with Fischer's medium (+4°C) and appropriate volumes of this cis-PAD solution were added as quickly as possible (ca 1 min) to 5 ml or 10 ml cultures containing $1-3 \times 10^5$ cells per ml. Control cells were treated with DMSO diluted 1:4 with Fischer's medium and this did not affect the cloning efficiency.

The cell suspensions were vigorously shaken after adding cis-PAD solution, exposed to the drug for 45 min at 37°C and subsequently transferred into test-tubes and centrifuged for 7 min at ca 500 g. The change of medium, carried out in the same sequence as adding cis-PAD solution, took place 60 min after the start of drug treatment; the sample temperature decreased during centrifugation to 30°C. Taking into account that half-life of biological activity of cis-PAD in culture media is close to 1 h [6], this procedure was considered equivalent to 1 h cis-PAD treatment at 37°C of monolayer cell cultures, when change of medium is much simpler.

In experiments involving cloning, immediate dilution of cis-PAD in treated samples made negligible the influence of trace amounts of the compound left during the medium change procedure. In all other types of experiments a second change of warm (37°C) Fischer's medium was carried out.

2.4. Viability determinations

Viability of the cell cultures was determined by nigrosine staining, according to the procedure described elsewhere [23].

2.5. Survival determinations

Survival of L5178Y cells was determined by cloning or by growth curve backward extrapolation [24].

The cloning technique for L5178Y-S cells was previously described in detail [25]. L5178Y-R do not form clones under these conditions, unless the medium used for cloning is supplemented with sodium pyruvate (BDH, Poole, UK) (100 µg/ml) [26] and β-mercaptoethanol [27] (Koch-Light, Colnbrook, UK) (3 µg/ml) immediately before making the final dilution of the cell culture. Cloning efficiency averaged 62 % for L5178Y-R cells and 75 % for L5178Y-S cells.

Some experiments were carried out according to a modified backward extrapolation method (kinetic method), as described in detail elsewhere [28].

The dose-survival curves were fitted by the least squares method; the data points are shown as mean values from at least three experiments per point ± standard error of the mean, if larger than the points plotted; otherwise, the individual values are shown.

2.6. DNA synthesis

DNA synthesis was examined by labelling of 10 ml cultures (2×10^5 cells), 15 min after completion of cis-PAD treatment, with [Me-^{3}H]thymidine (^{3}H-dThd; specific activity 624.5 MBq UVVVR, Czechoslovakia) at a concentration of 11.1 Bq for 1 h. Each culture was further divided into two 4 ml samples, in which the radioactivity of 5% trichloroacetic acid-insoluble fraction was measured by the liquid scintillation method [29]. Results were expressed in counts per min per 10^5 cells and then as percent of incorporation into the control cells.

2.7. Autoradiography

For autoradiography 5 ml cultures containing $2\text{-}4 \times 10^5$ cells were used. Pulse-labelling was performed at 37°C using ^{3}H-dThd (cf. 2.6 DNA synthesis) for 20 min at a concentration of 111 Bq/ml; continuous labelling was performed with 11.1 Bq/ml ^{3}H-dThd for 1-4 h.

After completion of the labelling, cell suspensions were transferred into test-tubes containing 5 ml of cold (+4°C) saline with dThd (70 mg/l) and centrifuged for 7 min at ca 300 g. Cell pellets formed were washed with 0.9% NaCl: 0.075 N KCl (1:1) (L5178Y-S cells) or 0.075 M KCl (L5178Y-R cells) (cf. Table 1, osmotic fragility). Cells were subsequently fixed twice with cold (+4°C) Carnoy's fixative (15 min and 24 h). Smears on gelatine-coated [30] microscopic slides were prepared, covered with Kodak AR10 stripping emulsion, exposed for 10 days at +4°C, developed according to [30], except that UNIFEN developer (Bydgoskie Zakłady Fotochemiczne, Bydgoszcz, Poland) and 1% acetic acid for rinsing were used, and stained by dipping for 5 sec in 10% Giemsa. For labelling index or mitotic index determinations 1000-2000 cells per slide were counted.

2.8. Chromosome preparations

For analyses of kariotypes and of chromosomal aberrations 5 ml cell cultures ($2\text{-}4 \times 10^5$ cells) were treated for 3 h with colchicine (Colchinéos, Houdé, Paris, France) (40 µg/ml) [31] at 37°C.

After centrifugation for 7 min at ca 300 g the cells were given a hypotonic treatment in 0.75 % sodium citrate for 27 min at room temperature (L5178Y-R cells) or in 0.75 % sodium citrate: 0.075 N KCl (1:1) for 12.5 min at room temperature (L5178Y-S cells). Time of the hypotonic treatment includes time of subsequent centrifugation. Cells were further fixed twice with cold (+4°C) Carnoy's fixative (15 min and 20 h), spread on chilled microscopic slides, dried over a micro-burner flame and stained with 10 % Giemsa for 1 h. Number of metaphases analyzed was 100-150 (chromosome number), 50-100 (chromatid aberrations, treated cells), or 25 (chromatid aberrations, control cells).

2.9. Cell cycle analysis

For determination of cell cycle parameters the method of continuous labelling with ^{3}H-dThd in presence of colchicine [32] was used. In control L5178Y-R and L5178Y-S cell duration of G2 and S phases was additionally determined by means of pulse labelling with ^{3}H-dThd followed by colchicine treatment, according to [33].

Progression through the cell cycle of cis-PAD treated L5178Y cells was studied using the continuous labelling + colchicine method. The label was given 15 min after completion of cis-PAD treatment of 40 ml cultures (i.e. immediately after washing the cells with Fischer's medium). Control cells were treated with DMSO only; this treatment did not disturb L5178Y cell progression through the cell cycle. Colchicine concentration was 40 µg/ml [31].

The labelling and preparation of autoradiograms are described under 2.7. "Autoradiography".

3. RESULTS

3.1. Characteristics of L5178Y murine lymphoma strains

A number of L5178Y cell strains have been used in various laboratories. In the work presented, the strains R and S used correspond to those designated R/F and S/F by Lett et al. [34].

A characteristic feature of the L5178Y-R strain is its ability to spontaneously transform into L5178Y-S strain after several months of cultivation in vitro. Therefore, L5178Y-R cells were passaged in vivo (DBA-2 mice, ip injection) every 2-3 months. The detailed characteristics of both L5178Y strains are compared in Table 1.

TABLE 1. Some characteristic features of L5178Y-R and L5178Y-S murine lymphoma strains. References in brackets.

	L5178Y-R	L5178Y-S
Cell diameter	14 µm [28]	16 µm [28]
Nucleus diameter	9 µm [35]	9 µm [35]
Modal chromosome number*)	40	39
DNA content per cell	$0.85 \pm 0.04 \times 10^{-8}$ mg	$0.93 \pm 0.4 \times 10^{-8}$ mg [36]
Protein content per cell	$1.25 \pm 0.13 \times 10^{-7}$ mg	$1.25 \pm 0.13 \times 10^{-7}$ mg
Growth in vitro:		
Doubling time	11-14 h	10-11 h
Non-cycling compartment (from 24 h labelling index)	none	none
Cell cycle parameters		
T_G*)	12.6 14.0	10.3
duration of: G1 (h)	2.1 3.0	1.6
S (h)	8.1 9.2	6.8
G2 (h)	1.6 1.4	1.5
M (h)	0.5 0.5	0.5
Cloning	in Fischer's medium containing 0.19 % agar, supplemented with sodium pyruvate and mercaptoethanol	in Fischer's medium containing 0.19 % agar
Growth in vivo: (after ip injection into DBA/2 mice)	ascitic tumour	solid tumour
Implantability	high	low
Osmotic fragility	low	high [37]

*) for comments, see text.

Similarly to many malignant cell lines grown in vitro, L5178Y cells undergo fluctuations in ploidy and duration of generation time. In fact, part of the dose-survival experiments was carried out, using a near-tetraploid L5178Y-R cell line, which arose spontaneously from a near-diploid one; no difference in susceptibility to cis-PAD was observed between this near-tetraploid line and the parental near--diploid one.

The near-tetraploid line was subsequently cloned, a near-diploid clone isolated and used in further experiments, among others, for studies on chromatid aberrations and for chromosome number determinations.

The DNA content was determined in near-diploid lines; the difference between values obtained for both strains is not significant by Student's test (8 df, $P = 0.95$).

The generation time (T_G) of L5178Y-R cells is also a subject to some variation, the prolongation being due to increase in G1 and S phase duration (Table 1). Most of the studies presented were carried out using a L5178Y-R cell line with T_G ca 12.6 h and a near--diploid karyotype. The other L5178Y-R line with T_G ca 14 h was also near-diploid and did not differ from the first line in respect to radiosensitivity and susceptibility to cis-PAD. T_G of L5178Y-S cells is less variable.

Numbers of chromosomes occuring in cells of both strains are given in Fig.2. Karyotype of L5178Y-S strain is more stable than that of L5178Y-R strain, although variable proportions of aneuploid cells were observed in the course of these studies. This, again, did not influence the susceptibility to cis-PAD treatment. Likewise, radiosensitivity remained unchanged.

As mentioned previously, L5178Y strains exhibit the unique feature of inverse cross-sensitivity to X-rays and UV-light. This property of L5178Y strains is illustrated by the survival curve parameters, presented in Table 2.

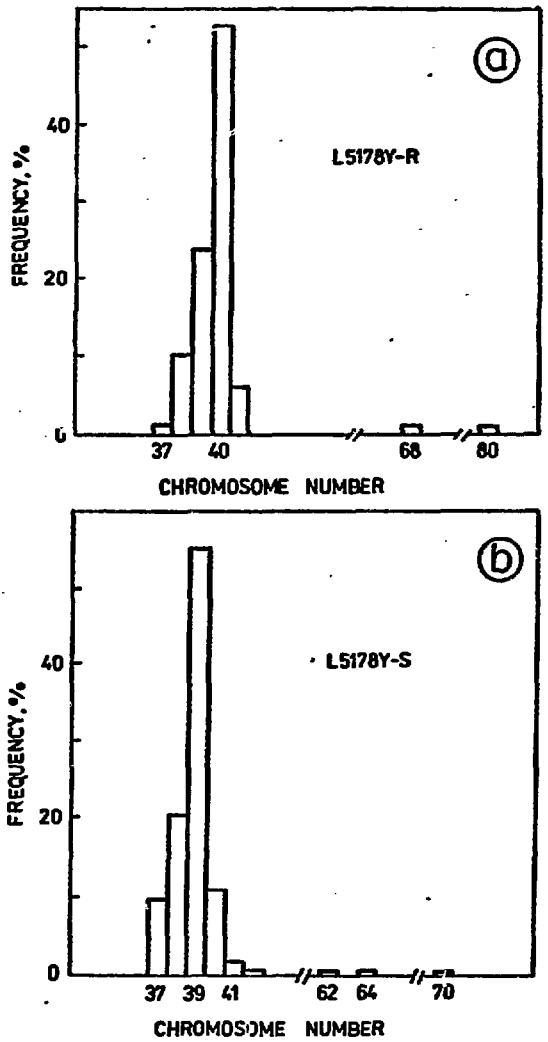


FIG. 2. Chromosome numbers in cells of L5178Y-R (a) and L5178Y-S (b) strains.

TABLE 2. Parameters of dose-survival curves of X- and UV-irradiated L5178Y cells.

Radiation [reference]	L5178Y-R			L5178Y-S		
	D_o	D_q	n	D_o	D_q	n
X [21]	0.91 Gy	0.40 Gy	1.5	0.56 Gy	0	1
UV [38]	3.23 J/m^2	0	1	3.98 J/m^2	3.96 J/m^2	1.5

3.2. Dose-survival relationships

The survival curves in Fig.3 show that the sensitivity of L5178Y-R and L5178Y-S cells to cis-PAD differs significantly. Two pairs of curves are shown for each cell strain: the lower curves are obtained by the kinetic backward extrapolation method, the upper ones by cloning. The difference in slopes indicates occurence of division delay. The data are presented without taking this delay into account.

The survival curves of L5178Y-R cells are exponential, with extrapolation number close to 1. The curves obtained for L5178Y-S cells are curvilinear; this type of dose-survival relationship is better expressed in terms of the molecular theory of survival [39,40,8]. However, for the sake of comparison with UV-irradiation effects, all the survival data in this work are presented using parameters introduced for the target theory.

Only a few experiments were carried out by the kinetic method. Some of the points plotted in Fig.3 derive from single determinations. Therefore, the survival curve obtained by the kinetic method for L5178Y-S cells was drawn by eye and standard errors were not indicated. The parameters of dose-survival curves were calculated for both strains using survival data from the cloning experiments.

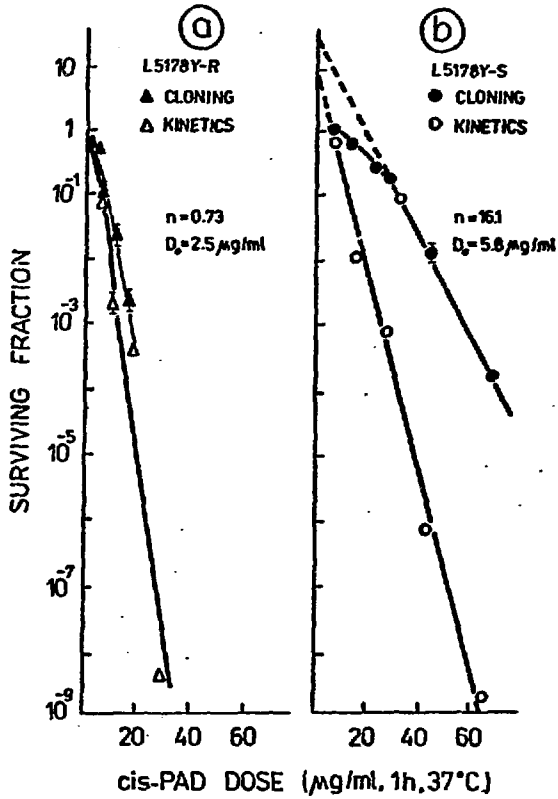


FIG.3. Dose-survival curves of cis-PAD - treated L5178Y-R (a) and L5178Y-S (b) cells.

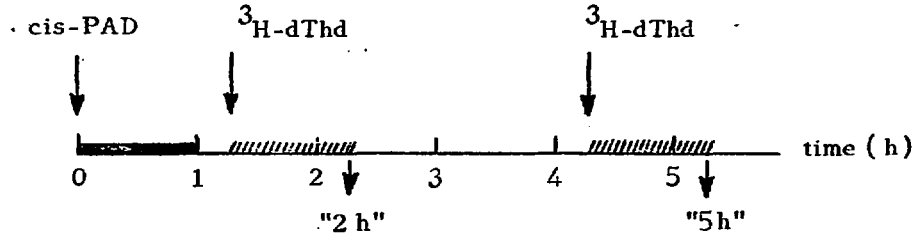
3.3. DNA synthesis

cis-PAD, similarly to other platinum complexes, inhibits DNA synthesis [6]. Decrease of incorporation of ^{3}H-dThd was observed in both L5178Y strains and was due to slower replication of DNA, rather than to a change in the number of DNA-synthesizing cells. Such a conclusion could be drawn from the comparison of "2h" labelling indices (LI) (Table 3) with the incorporation data (cf. Fig.11c): decrease of incorporation of ^{3}H-dThd into DNA after cis-PAD treatment was influenced only slightly by the small decrease in percentage of DNA--synthesizing cells. Using LI "2h" values, the incorporation data were corrected for the percentage of DNA-synthesizing cells.

TABLE 3. Labelling indices in cis-PAD-treated L5178Y-R and L5178Y-S cells (mean from 2-7 experiments ± SE, or values from single determination).

L5178Y strain	cis-PAD dose (μg/ml, 1 h, 37°)	Labelling index (%)	
		"2h"	"5h"
R	0 (control)	67.0 ± 0.7	70.1 ± 2.5
	4.0	66.3 ± 1.9	70.3 ± 0.5
	7.8	66.3 ± 1.7	70.4 ± 1.3
	15.4	66.9 ± 1.9	71.2 ± 0.7
	42.8	63.3 ± 3.1	65.0 ± 0.5
S	0	71.9 ± 1.6	70.3
	4.0	69.8 ± 2.2	69.0
	7.8	69.0 ± 0.3	70.3 ± 2.4
	15.4	66.3 ± 1.9	69.5 ± 2.4
	42.8	64.5 ± 0.9	70.4 ± 2.0

Experimental procedure:



The corrected data for cis-PAD-treated L5178Y-R and L5178Y-S cells are shown in Fig.4; the difference found at the highest cis-PAD dose is statistically significant by Student's test (6 d.f. P 0.99). Thus, from the point of view of DNA synthesis inhibition L5178Y-R strain is more sensitive to cis-PAD than L5178Y-S strain. For further comments to Table 3, see "3.5. Progression through the cell cycle".

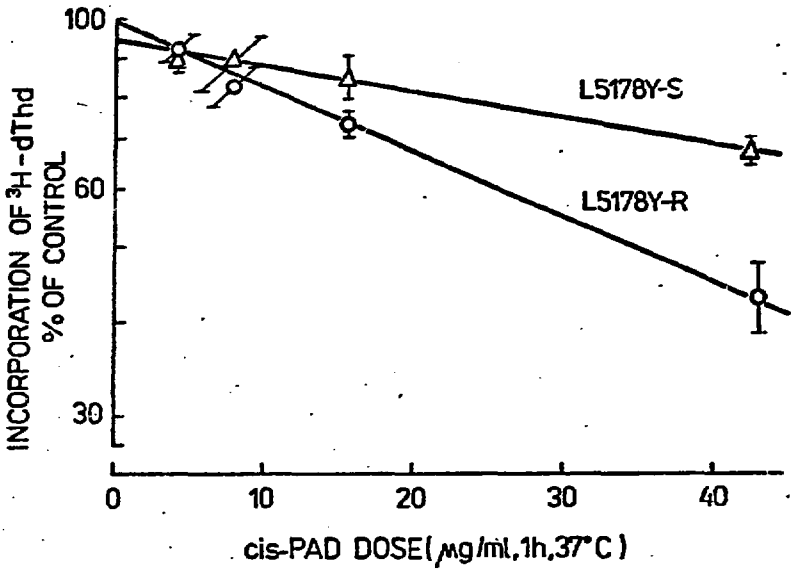


FIG.4. Incorporation of ^{3}H-thymidine into DNA of cis-PAD-treated L5178Y cells. The incorporation data were corrected for the percentage of cells synthesizing DNA.

3.4. Chromatid aberrations

Formation of chromatid aberrations was studied, using L5178Y-R and L5178Y-S cultures treated with cis-PAD, 15.4 and 42.8 μg/ml, respectively, for 1 h at 37°C. Survival of both strains studied is reduced by such treatment with cis-PAD to ca 0.3 %. The design of the experiment is shown in Fig.5 together with the mitotic indices. Pooled data obtained in two experiments are presented in Table 4.

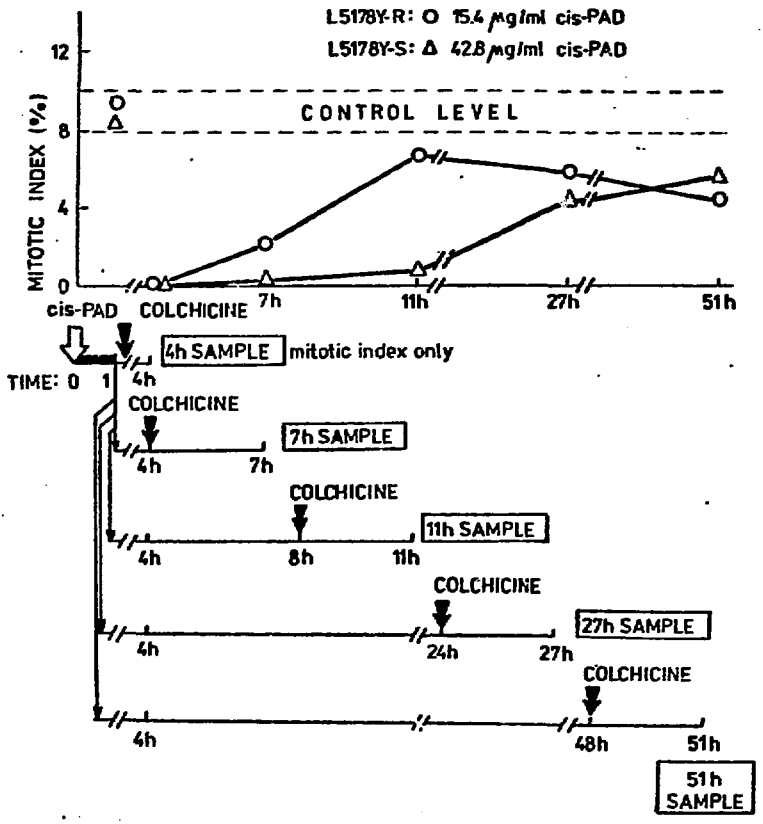


FIG.5. Diagram of L5178Y cell treatment for studies on chromatid aberrations; mitotic index values in the respective colchicine-treated samples are shown in the upper part of the graph.

TABLE 4. Chromatid aberrations in L5178Y cells treated with cis-PAD.

Cell strain	cis-PAD dose and survival	Time after cis-PAD treatment (h)	Number of metaphases analysed	Percent of metaphases without chromatid aberrations	Percent of metaphases with degenerated chromosomes	Chromatid aberrations per 100 cells	
						Breaks	Exchanges
L5178Y-R	0 (control)	7	25				
		11	25	100	0	< 1	< 1
	1	27	25				
		51	25				
	15.4 µg/ml 1 h, 37°C	7	25	100	0	0	0
		11	50	90	0	3	4
	2.3×10^{-3}	27	100	7	49	62	95
		51	75	32	53	23	32
L5178Y-S	0 (control)	7	25				
		11	25				
	1	27	25	100	0	1	1
		51	25				
	42.8 µg/ml 1 h, 37°C	7	4*	100*	0	0	0
		11	79	93.7	0	6.3	2.5
	4.5×10^{-3}	27	70	22.9	10.0	37.1	102.8
		51	75	42.7	25.3	54.7	36.0

* mitotic index < 0.01 %.

The delayed appearance of chromatid aberrations after cis-PAD treatment previously described for synchronized CHO population [6] is clearly seen. 11 h after treatment only a few percent of abnormal metaphases were observed; 27 h after treatment, apart from numerous chromatid aberrations, in a considerable number of metaphases "degenerated" or completely "shattered" chromosomes were seen, similarly to Chinese hamster cells after treatment with cis-DDP [41]. Also, in many apparently normal metaphases, a characteristic pattern of chromosome arrangement was found, due to the phenomenon of "sticky ends".

3.5. Progression through the cell cycle

For studying the effect of cis-PAD treatment on progression of L5178Y cells through the cell cycle the continuous labelling + colchicine method of Puck and Steffen [32] was applied. In Fig.6 the change of the G1 phase duration in cis-PAD (30 μg/ml, 1 h 37°C) - treated cells is presented in the graphical form introduced by Maekawa and Tsuchiya [33]. Similarly to CHO cells [6], in L5178Y cells a prolongation of G1 phase is observed after cis-PAD treatment.

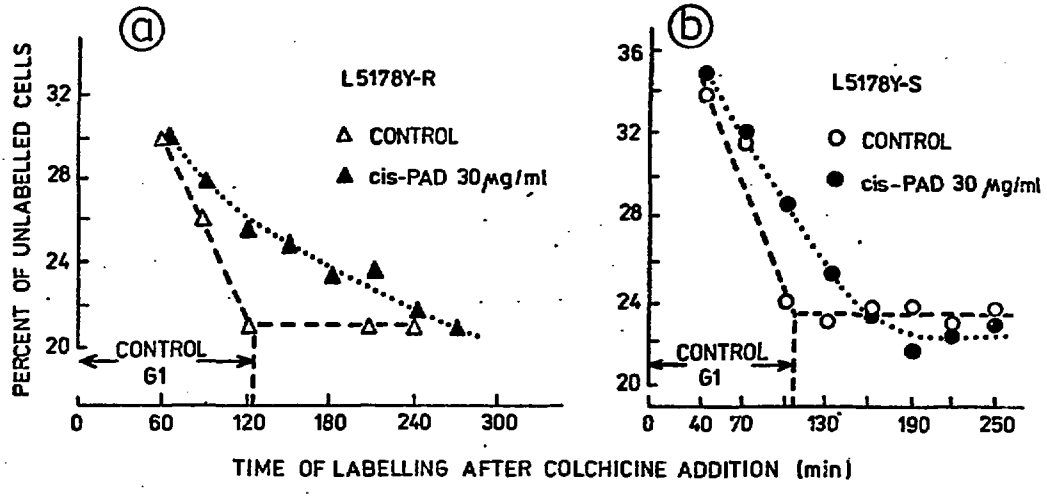


FIG.6. Duration of G1 phase in control and cis-PAD-treated (30 μg/ml, 1 h, 37°C) L5178Y-R (a) and L5178Y-S (b) cells, determined according to [33]. Colchicine concentration was 40 μg/ml [31].

This probably accounts for the slight decrease in the labelling index, seen 1-2 h after drug treatment (cf. Table 3, "2 h" LI).

There is also a delay in entrance of G2 cells into mitosis, as indicated by the plot of collection function log (1+N/M) [32] versus time, where N(M) is the fraction of mitotic cells (Fig.7). The effect is almost immediate after treatment and both cell strains respond similarly, resembling in this respect Ehrlich ascites cells and chick embryo fibroblasts [42,43]. Although the term "mitotic delay" has been used further in the text, this is not intended to mean that the pre-mitotic death is excluded.

The "5 h" labelling index values tabulated in Table 3 do not differ significantly enough from the control values to indicate a retention in S phase at this post-treatment stage of cis-PAD-treated L5178Y cell populations.

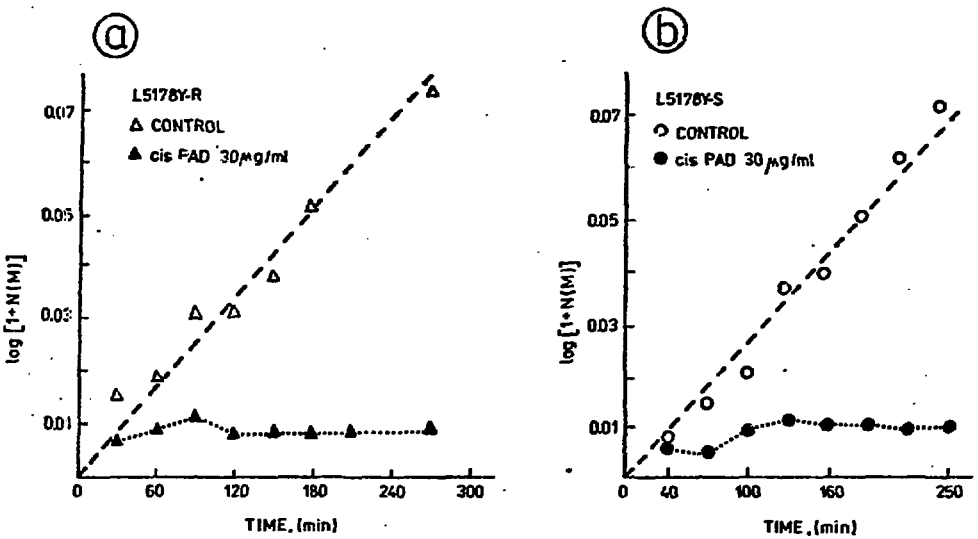


FIG.7. Plot of the collection function [32] log(1+N/M) for cis-PAD-treated (30 µg/ml, 1 h, 37°C) L5178Y-R (a) and L5178Y-S (b) cells. Colchicine concentration was 40 µg/ml [31].

3.6. Growth and viability

Observations on growth and viability were carried on L5178Y-R and L5178Y-S cultures treated with doses of cis-PAD reducing survival of both strains to comparable levels in the range from 6×10^{-1} to 2×10^{-9}.

Examples of growth curves are given in Fig.8.

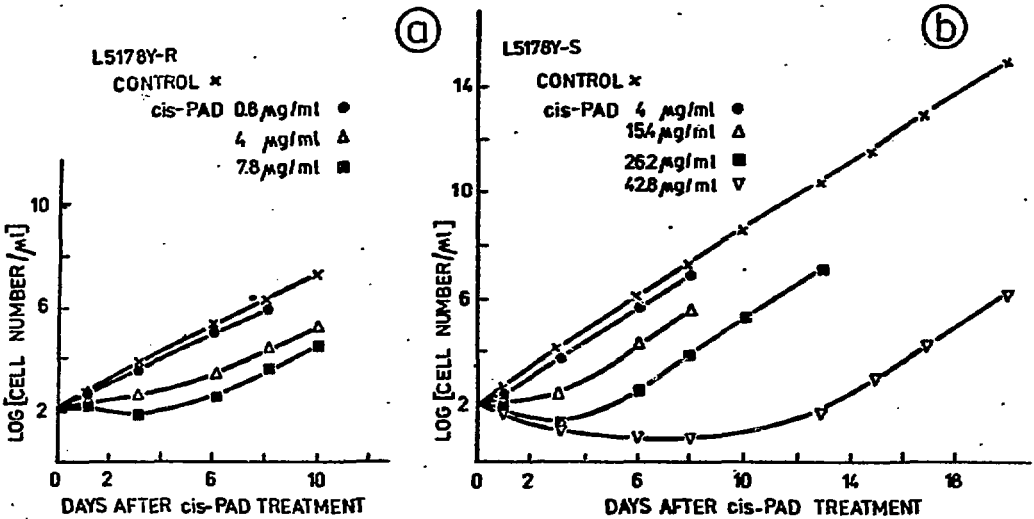


FIG.8. Growth curves of cis-PAD-treated L5178Y-R (a) and L5178Y-S (b) cells.

The same growth curves are also presented in Fig.9 using a plot of $N_T : N_C$ versus time after treatment; $N_T : N_C$ is the ratio of the number of cells in the treated population to the number of cells in the control [44]. This graphical system makes it possible to visualise more clearly growth disturbances. The viability changes are plotted on the same graph.

As can be seen, for cis-PAD-treated cell populations a phase I - phase III growth pattern can be distinguished [44,45]: the period of serious growth disturbances and low viability (phase I) directly passes into the period of proliferation at the normal rate (phase III). The decrease in viability is clearly dose-dependent and the period of its occurence coincides with the appearance of chromatid aberrations (cf.3.4). After elimination of the lethally damaged cells (and possibly some non-lethally damaged ones) normal growth is resumed.

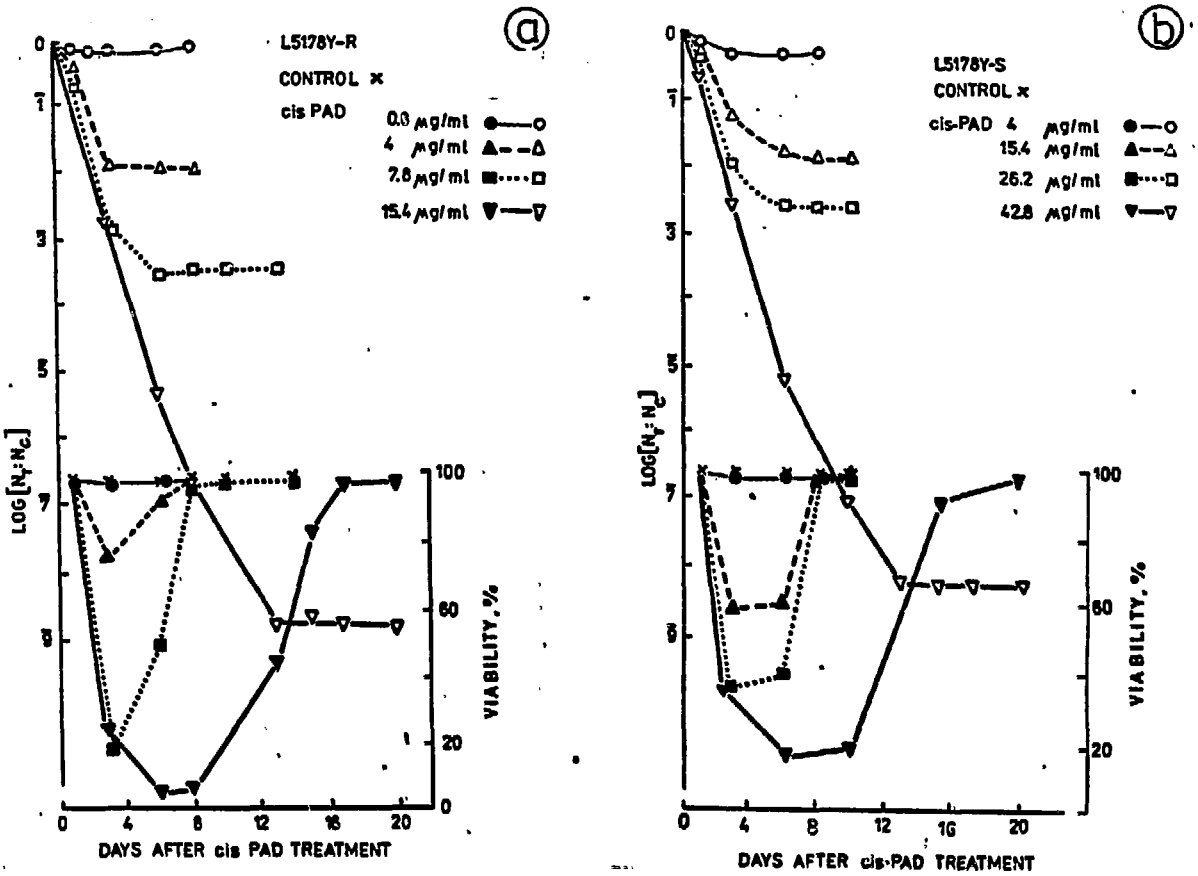


FIG.9. Growth patterns and viability changes of L5178Y-R (a) and L5178Y-S (b) cells. For explanation, see text.

4. DISCUSSION

The data on cytotoxic action of cis-PAD indicate that all the essential features of the action of this platinum complex on CHO cells [6] such as immediate inhibition of progression through the cell cycle, reduced rate of DNA synthesis, delayed appearance of chromatid aberrations and delayed death, have been found also in L5178Y cell strains studied. Such effects are usually observed when cells of a proliferating population are dying as a result of DNA damage. According to Bender et al. [46-48] different kinds of lesions in DNA can lead to the same type of chromosomal damage; although the lesions inflicted by various DNA-damaging agents manifest themselves differently at the cellular level, the ultimate cause of death seems to be the loss of reproductive ability.

While chromosomal damage is regarded by many authors as the cause of reproductive death, directly related to lethality [49-51,8,40], abnormalities of karyokinesis [52] or unbalanced growth of cells [53,54] are indicated by other investigators as additional causes of this kind of death.

Comparison of lethal and non-lethal effects of DNA-damaging agents on mammalian cells can provide information on consequences at the cellular level of certain types of DNA damage, as well as on the repair mechanisms. With this assumption, a detailed comparison of cis-PAD, X-ray and UV-light effects on L5178Y cell strains is presented below.

4.1. Sensitivity

There is considerable evidence that cytotoxic action of cis-dichlorobis(amine)platinum(II)complexes depends on selective binding to DNA and forming inter- and intra-strand cross-links [9,11,17]. The replication pattern observed for the best-known and most studied complex - cis-dichlorodiamineplatinum(II)(cis-DDP) [55] suggests that effects of this complex on DNA replication are comparable with those of UV-light [56].

No data on sedimentation of newly syntesized DNA of cis-PAD-treated L5178Y cells are available. However, a striking similarity in L5178Y-R and L5178Y-S cell response to cis-PAD and UV-light can be seen at the cellular level. The respective survival curves [38] are presented in Fig.10. The only significant difference is in extrapolation numbers of cis-PAD-treated and UV-irradiated L5178Y-S cells.

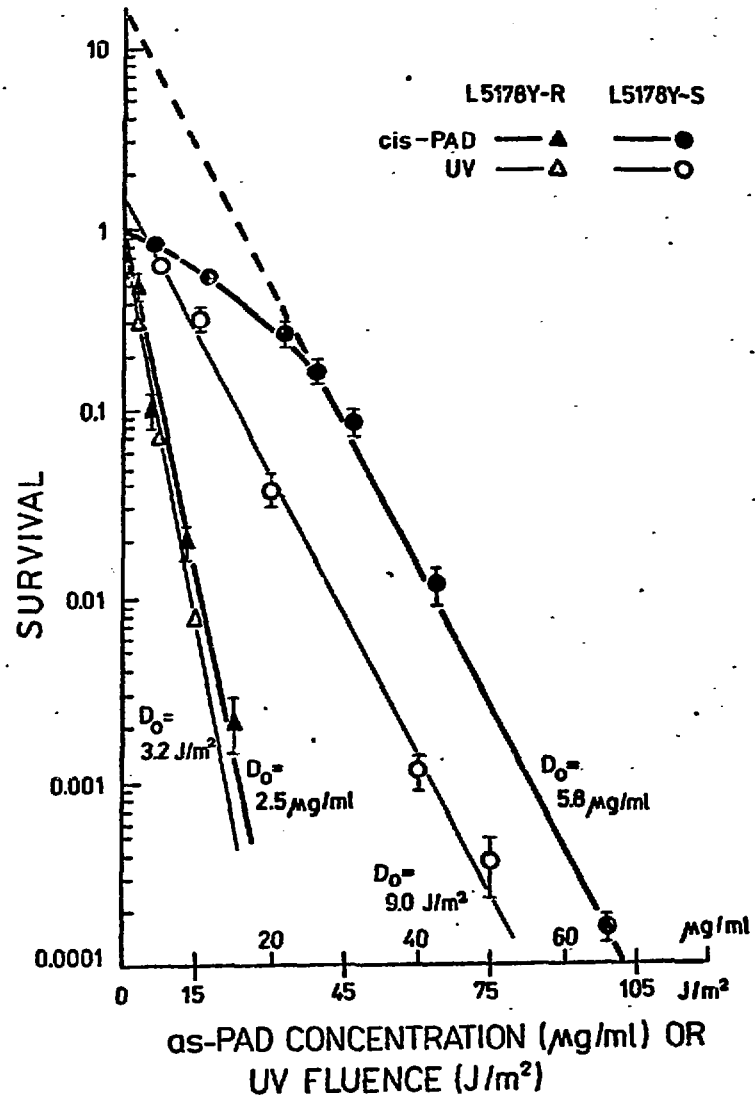


FIG. 10. Comparison of survival curves of cis-PAD-treated and UV-irradiated L5178Y cells.

Such close resemblance of dose-survival relationships suggests that further comparisons of cis-PAD and UV-light effects can aid understanding of the mechanisms underlying sensitivity to cis-PAD alone and in combination with radiation.

4.2. DNA synthesis

Incorporation of ^{3}H-dThd into DNA of cells treated with DNA--damaging agents is often taken as a measure of the extent of damage produced in the replicative template [57]. In the case of X-irradiation it can also be a reliable indicator of the cell sensitivity. [58,54].

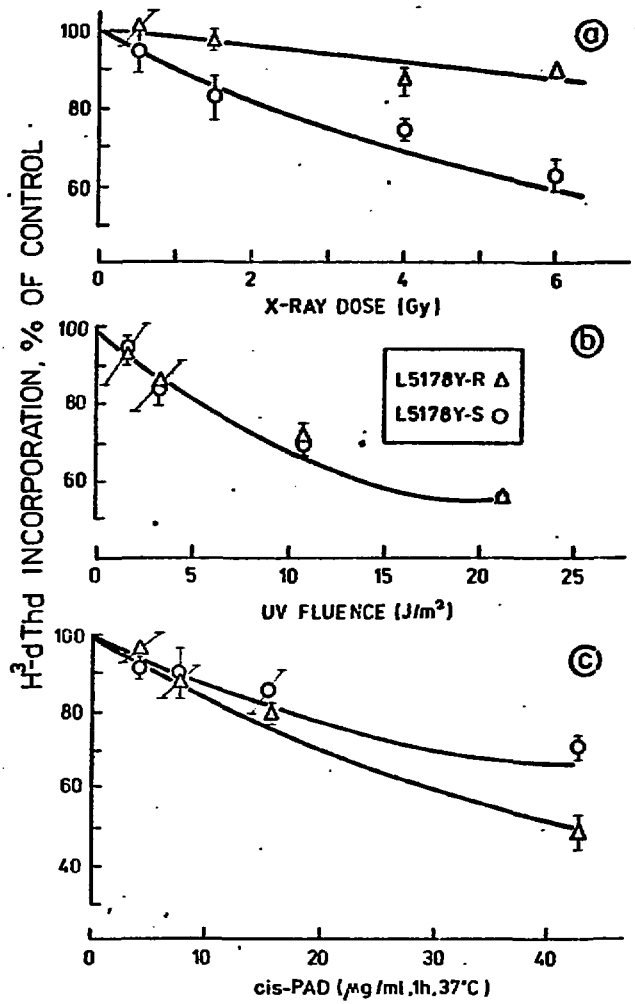


FIG. 11. Comparison of ^{3}H-dThd incorporation in L5178Y cells treated with various DNA-damaging agents: a) X-rays, b) UV-light, c) cis-PAD.

As shown in Fig.11 a L5178Y strains distinctly differ in ^{3}H-dThd incorporation after X-irradiation, the radiosensitive L5178Y-S strain being more affected (cf. D_o values, Table 2). In contrast to X-rays, UV-light causes a similar effect in both strains (Fig.11 b); the difference in response to cis-PAD is less pronounced than that observed after X-irradiation, especially at lower doses. Although the initial amount of damage inflicted by UV-light seems to be the same, or nearly the same in both L5178Y strains, its expression at the cellular level significantly differs, as may be seen from the dose-survival relationship (cf. Fig.10, Table 2). The same applies to a lesser extent to the cis-PAD effect in both L5178Y strains.

4.3. Chromatid aberrations

DNA-damaging factors such as UV-light, alkylating agents and nitroso compounds produce, according to Bender et al. [46,49], chromatid breaks as a result of DNA synthesis on the damaged template. Hence, aberration production is of delayed type and only chromatid aberrations are found. The data described previously for cis-PAD-treated G1 CHO cells [5], as well as those obtained for L5178Y cells indicate that cis-PAD can be classified together with the above-mentioned DNA-damaging factors from the point of view of chromosomal aberration production.

The proportion of DNA lesions converted into chromatid aberrations seems to be directly related to the cell survival in both L5178Y strains: aberration yields (Table 4) are similar at cis-PAD doses reducing survival to ca. 0.3 %. These doses differ by a factor of 2.78. The only exceptions are much higher yield of chromatid breaks in L5178Y-R cells, 27 h after cis-PAD treatment, and higher number of metaphases with "degenerated" chromosomes in L5178Y-R than in L5178Y-S strain.

The apparent correlation between the frequency of chromatid aberrations and lethal effect of cis-PAD in L5178Y strains resembles that observed by Scott et al. [59] in two Yoshida strains differing in sensitivity to sulphur mustard. In the latter system drug resistance was accompanied by a reduced amount of induced chromosome damage. These findings might be explained by reverse relations between proportion of DNA lesions converted into chromosomal aberrations and efficiency of DNA repair processes. However, attempts to correlate cellular sensitivity and the extent of DNA repair failed in cells treated with alkylating agents [59,60] as well as in several

X-irradiated cell systems [61-64], although they were successful in UV-irradiated cells [65] and in X-irradiated murine lymphoma L5178Y [66]. Therefore, apart from DNA repair processes, hypothetical recovery processes influencing the proportion of DNA lesions converted into chromosomal aberrations, can be taken into consideration when causes of cellular sensitivity to DNA-damaging agents are analysed.

4.4. Mitotic delay

In some cell lines duration of mitotic delay is directly related to cell's ability to recover from the inflicted damage. At the same time a reverse relationship has been observed in other cells. In the first case an inhibition of progression through the cell cycle can be considered as a manifestation of a defence mechanism, allowing more time for repair processes before the entry into mitosis [67]. In the latter case the delay can be interpreted as a symptom of damage portending extensive cell death. The latter possibility was favoured by Ehmann et al. [52] who found in X-irradiated L5178Y-S/S cells an exceptionally long mitotic delay (ca 10 h/1 Gy), related to the extremely high radiosensitivity of this strain. A clear reverse correlation exists between radiosensitivity and duration of G2 block in several L5178Y sublines investigated by Rosenberg et al. [53]: lower D_o values were associated with longer duration of G2 block. The significance of this correlation is not clear: time-lapse photography indicates that the earlier an irradiated L5178Y-S/S cell divides, the higher ability it has of undergoing subsequent divisions; on the other hand - the earlier the cells reach mitosis after irradiation, the higher is the number of chromosomal aberrations exhibited [52], which is just the reverse of what could be expected from the ability to divide and of what was observed by other authors in various cell lines (cf. [68]).

In accordance with the correlation described by Rosenberg et al. [53], in the radioresistant L5178Y-R strain the duration of mitotic delay is ca 4 h/1 Gy, while in L5178Y-S strain - nearly 8 h/1 Gy ([69] and our own results). The mitotic delay in UV-irradiated L5178Y strains was not determined; the data available allow, however, to compare mitotic delays in cis-PAD-treated L5178Y cells. Using the formula of Rosenberg et al. [53] (cf. Table 5) for sensitivity in terms of mitotic delay (S_d) the following values were obtained: 0.0243 µg/ml (L5178Y-R strain) and 0.0245 µg/ml (L5178Y-S strain). Clearly, the delay in cis-PAD-treated L5178Y cells is dose-dependent

(cf. Fig.5), while not directly related to the cell survival: duration of mitotic delay in R and S strains differed by a factor of ca 2 at the same survival level. From the S_d values conclusion can be drawn that in the case of cis-PAD-treated L5178Y cells no direct relationship can be found between sensitivity in terms of mitotic delay and cell lethality.

4.5. Growth

Growth patterns were studied in detail in X-irradiated L5178Y-S cells [44,45] as well as in UV-irradiated L5178Y cell strains [28,70]. X-irradiation was found to induce frequently growth retardation caused by damages transmissible to progenial cell generations [45]. Growth stimulation observed often after UV-irradiation [28,70] can be ascribed to survival of the most fit cells in the population. cis-PAD, unlike ionizing radiation, shows no tendency to induce non-lethal lesions in L5178Y cell strains and, from this point of view, resembles UV-light.

4.6. Concluding remarks

Similarities and differences in response to cis-PAD, X-rays and UV light of two L5178Y strains are summarized in Table 5. The comparison of L5178Y cell response to the three DNA-damaging agents suggests that there are some similarities in sensitivity to UV-light and cis-PAD. This finding could be explained on the assumption that UV-light and cis-PAD induce lesions which are eliminated by the same type of repair processes and that L5178Y strains differ in ability to perform such repair. An alternative explanation would be that similarities in response to cis-PAD and UV-light are fortuitous and that the lesion types and repair processes operating after treatment with these agents have little in common; the difference in sensitivity of both strains to cis-PAD could then be due to a difference in uptake and intracellular binding of cis-PAD and/or to efficiency of repair processes specific for cis-PAD-induced lesions. These alternatives have been examined and the results obtained are the subject of the following papers in this series [71,72].

TABLE 5. Similarities and differences in response of L5178Y-R and L5178Y-S strains to DNA-damaging agents.

Type of response	DNA-damaging agent (dose units in brackets)		
	cis-PAD (μg/ml, 1 h, 37°C)	UV (J/m^2)	X (Gy)
Sensitivity in terms of cell killing			
$1/LD_{90}$	R: 0.20 S: 0.034	R: 0.14 S: 0.042	R: 1.0 S: 8.0
$1/D_o$	R: 0.40 S: 0.17	R: 0.31 S: 0.11	R: 0.99 S: 1.85
	$\sigma_R > \sigma_S$	$\sigma_R > \sigma_S$	$\sigma_R < \sigma_S$
Sensitivity in terms of mitotic delay, S_d	R: 0.024 S: 0.024	not determined	R: 0.003 S: 0.008
	$\sigma_R = \sigma_S$		$\sigma_R < \sigma_S$
Inhibition of DNA synthesis			
$1/LD_{90}$	R: 0.17 S: 0.11	R: 0.52 S: 0.52	R: 0.22 S: 1.00
	$\sigma_R > \sigma_S$	$\sigma_R = \sigma_S$	$\sigma_R < \sigma_S$
Growth after elimination of dead cells from the population	at the normal rate	stimulated or at the normal rate	retarded or at the normal rate

LD_{90} – dose which kills 90 % of treated cells

S_d – $\frac{\text{mitotic delay (h)}}{\text{generation time (control) (h) x dose}}$ [53]

ID_{90} – inhibitory dose reducing ^{3}H-dThd incorporation to 90 % of the control incorporation

σ_R, σ_S – sensitivities of L5178Y-R and L5178Y-S strains, respectively.

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