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Effects of Norcantharidin, a Protein Phosphatase Type-2A Inhibitor, on the Growth of Normal and Malignant Haemopoietic Cells

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Cantharidin is a natural toxin that inhibits protein phosphatase type 2A (PP2A) and has antitumour effects in man. We have studied the synthetic analogue, norcantharidin (NCTD), which has less nephrotoxic and phlogogenic side-effects, investigating the effects on the normal haemopoietic system and leukaemia cell growth. Daily intraperitoneal (i.p.) injection of NCTD induced dose and circadian time-dependent transient leucocytosis in normal mice, but did not accelerate bone marrow (BM) regeneration, or have haemopoietic side-effects following chronic administration. NCTD stimulated the cell cycle progression of granulocyte–macrophage colony-forming cells (GM-CFC), stimulated DNA synthesis and increased the frequency of mitotic cells in short-term human BM cultures. NCTD also stimulated the production of interleukin (IL)-1β, colony stimulating activity (CSA) and tumour necrosis factor (TNF)-α. Continuous *in vitro* NCTD treatment, however, inhibited both DNA synthesis and GM-CFC growth. Fluorescence-activated cell sorting (FACS) analysis of DNA profiles and cytological studies in HL-60, K-562 or MRC5V2 (fibroblast) cells indicated that low doses of NCTD accelerated the G_1/S phase transition, while higher doses or prolonged incubations inhibited the cell cycle at the G_2/M phases or during the formation of postmitotic daughter cells. Electron microscopy revealed that NCTD impaired the neogenesis of chromatin material and nuclear membrane during the M/G_1 phase transition in K-562 cells. The biphasic effect of NCTD may be due to inhibition of PP2A activity, which regulates the cell cycle, both at the restriction point and at the G2 and M phases. Our data provide new insight into the cellular and molecular actions of NCTD, and partly explain its therapeutical effects in cancer patients.

Key words: norcantharidin, protein phosphatase 2A inhibitor, leucocytosis, cytokines, cell cycle control, stem cells, leukaemia

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INTRODUCTION

SEVERAL SPECIES of blistering beetle, the Meloidae (*Cantharis vesicatoria* or *Mylabris cichorii* L.) have been recognised for many centuries for their aphrodisiac, phlogistic, vesicant and toxic effects. Dried, powdered beetle, mylabris, has been used to treat furuncles and piles, deep ulcers, venomous worms and fistulae of tuberculous lymphadenitis. The first documented use of mylabris to treat cancer, by the physician Yang Shi-Ying, dates back to 1264 [1].

Cantharidin (Figure 1a), the active substance of mylabris, inhibits some tumour cell types (HeLa cells, murine ascites hepatoma or reticulocell sarcoma) but not others (murine erythroid leukaemia cells *in vitro*, S180 *in vivo* and Walker tumour in rats) [1]. Clinical trials indicated that cantharidin had effects on patients with primary hepatoma, but the application was limited by its severe toxicity for mucous membranes, mainly in the gastrointestinal tract, ureter and kidney [1]. A series of bioactive analogues has been synthesised by Wang and associates over the past 10 years, in an attempt to increase the utility and to reduce the toxic side-effects of cantharidin. Norcantharidin (NCTD), the demethylated analogue of cantharidin (Figure 1b), appeared to cause the least nephrotoxic and inflammatory side-effects. The acute LD_{50} of NCTD is 11-fold higher than cantharidin (12.5 mg/kg). NCTD inhibits the proliferation of several tumour cell lines (HeLa, CHO, CaEs-17, BEL-7402, SMMC7721 human hepatoma, HEP-2 and human epidermoid laryngocarcinoma) and transplanted tumours (embryonal adenocarcinoma or hepatoma 22 in mice) [1, 2].

The exact molecular and cellular effects of NCTD have not yet been elucidated. Cantharidin, the parent compound of NCTD, is one of the three 7-(oxabicyclo-3.2.1.)heptane-2, 3-dicarboxylic acid derivatives; the others are the herbicide endothall (Figure 1c) and endothall thioanhydride. Each of these structurally related toxins, as well as the polyether fatty acid, okadaic acid, isolated from the marine sponges *Halichondria okadaic*, form stable complexes with protein phosphatase 2A (PP2A) [3, 4]. PP2A is a critical member of the PP family which

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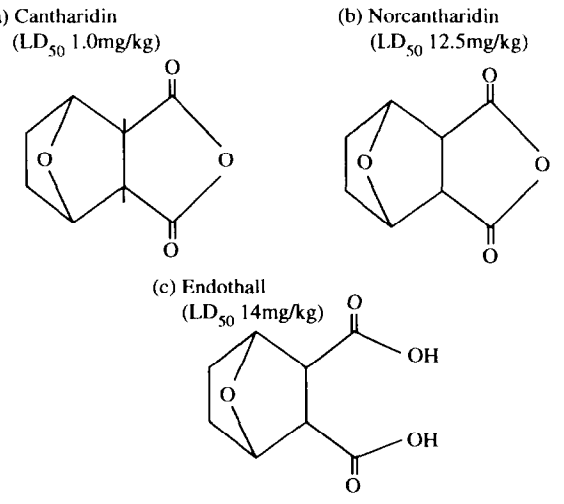

Figure 1. Chemical structure and LD_{50} in mice of the natural protein phosphatase type 2A (PP2A) inhibitor, cantharidin (a), the synthetic analogue, norcantharidin (b) and the structurally related plant toxin, endothall (c).

is widely distributed in the plant and animal kingdoms, and is implicated in the reversible modulation of a number of cell functions.

While NCTD has been being used in cancer therapy in China since 1984, the available data do not explain either its growth inhibitory effect in tumour tissues [1] or its leucocytosis stimulatory effect in normal organisms. This study was, therefore, carried out to obtain new information on the effect of NCTD on haemopoietic cell renewal. We show that NCTD acts either directly or via the induction of cytokines by bone marrow (BM) stromal cells. Continuous *in vitro* treatment or a higher dose of NCTD inhibits the proliferation of tumour cells by impairing chromatin organisation so that cells are arrested at the G_2/M or M/G_1 phase boundary and then die.

MATERIALS AND METHODS

Human BM aspirates

Samples of BM (3–5 ml) were aspirated from the sternums of healthy donors into 100 U preservative-free heparin, the donors having been advised of the risk of marrow aspiration in accordance with SMST guidelines and given their informed consent. The samples were diluted (1:1) with phosphate-buffered saline (PBS), and the low-density floating layers containing haematons, buffy coat (BC) cells and the mononuclear cell (MNC) fractions were isolated on Ficoll–Hypaque density gradients (d = 1.077g cm^{-3}) [5].

Murine BM cells

Male, 8-week-old DBA/2 or 8–12-week-old CBA×C57BL/F1 mice (IFFA CREDO L'Arbresle, France) were housed using a 12-h light–12-h dark lighting schedule. Food and water were provided *ad libitum*. The synchronisation of groups of mice was monitored by measuring rectal temperature, body weight and peripheral white blood cell (WBC) counts [6]. The effects of NCTD on the WBC counts and BM cellularity were determined by treating normal mice once a day with NCTD at the acrophase of the WBC count (6 h after light onset, HALO). Peripheral WBC and BM cells were analysed at the acrophase and at the nadir (15 HALO). Mice were injected intravenously (i.v.) once with 150 mg/kg 5-fluorouracil (5-FU) 24 h prior to the daily intraperitoneal (i.p.) administration of 2 mg/kg NCTD over 5 consecutive days. A BM plug was recovered from the femur of control and treated mice. The homogenous supernatant (BC) and the particulate sediment (haematon) fractions were analysed separately.

Organotypic haematon microcultures

The haematon fractions were purified by three repeated washes [7], and individual haematons were placed in 96-well round-bottomed plastic culture dishes to establish liquid cultures, or in 35-mm Petri dishes (Nunclon, Denmark) containing complete methylcellulose medium (Ready Mix, Terry Fox Laboratories, Vancouver, Canada) to establish semisolid cultures. The complete liquid culture medium consisted of Iscove's Modified Dulbecco's Medium (IMDM) (Flow, U.K.) supplemented with 0.5 mg/l ascorbic acid, 37 mg/l myoinositol, 10 mg/l folic acid, 50 μM 2-mercaptoethanol, 1 μM hydrocortisone hemisuccinate (Sigma, St Louis, Missouri, U.S.A.), 15% horse serum (Eurobio, France), 10% fetal calf serum (FCS) (Seromed, France) and antibiotics. The constitutive emergence of cells from human or murine haematons was measured by cell counting and by analysing clonogenic progenitor cells in secondary semisolid cultures. The adherent layer was analysed under a stereomicroscope on day 7 to determine the number of fibroblast colony-forming units (CFU-F) or cobblestone area-forming cells (CAFC) [5, 7].

Long-term liquid BM cultures (LTBMC)

Human LTBMCs were established with 10^6 BC cells/ml, or 10^6 cells/ml obtained from haematons dissociated with collagenase/dispase (Boehringer-Mannheim, Germany) at 37°C for 2 h, in 5 ml medium in 25 cm^2 tissue culture flasks (Nunclon). The cultures were agitated gently prior to harvesting non-adherent cells and spent medium. Cell-free supernatants were aliquoted and stored at −80°C until assayed for cytokines.

Haemopoietic colony-forming unit assays

High proliferative potential murine progenitor cells (HPP-CFC) were scored by a modification of the double layer technique [8]. An agar underlayer was first established with 0.7 ml 0.5% agar medium, containing a mixture of recombinant human growth factors (HGF): 50 U human IL-1, 4 ng mouse IL-3, 50 U mouse-macrophage colony-stimulating factor (M-CSF), 20 ng mouse granulocyte–macrophage CSF (mGM-CSF) (Genzyme, Tebu, France) and 20 ng human granulocyte-CSF (G-CSF; generous gifts from H. Takaku, Tokyo University, Japan). Test cells (10^5) were overlayered in 1.3 ml 0.8% methylcellulose medium containing 1% bovine serum albumin (BSA), 100 μM 2-mercaptoethanol, 1 mg/l transferrin, 100 mg/l soybean lecithin and 30% FCS (Ready Mix). The cultures were incubated at 37°C in humidified air containing 5% CO_2. Low proliferative potential GM-CFU populations were determined on day 14. Colonies larger than 1 mm in diameter were scored as HPP-CFC (high proliferative potential colony forming cell) on day 28. BFU-E (burst forming unit-erythroid) progenitor cells were cultured in methylcellulose medium containing the above HGFs and 2U erythropoietin (Epo) per ml and they were scored on day 14. Human HPP-CFC and GM-CFU populations were studied in the presence of recombinant human HGFs under the same conditions.

Permanent cell lines

Human leukaemic cell lines HL-60, K-562 and a normal fibroblast cell line MRC5V2 (gift from B. Azzaron, Villejuif,

France) were cultured in RPMI-1640 medium supplemented with 10% FCS and antibiotics. The cells were expanded to 1–6 × 10^5 cells per ml and their response to NCTD was studied during the logarithmic growth phase.

Yeast cell cultures

Wild-type baker's yeast, *Saccharomyces cerevisiae*, obtained from mass cultures (Sigma, St Louis, Missouri, U.S.A.), was used. The cells were washed in minimal, glucose- and nitrogen-free medium, and then synchronised at 25°C for 180 min. Washed cells were resuspended in complete medium containing glucose and nitrogen (YDP, Difco, Michigan, U.S.A.). The cells were treated with increasing concentrations of NCTD at 25°C. The kinetics of cell cycle progression was analysed by cell counting and on fixed and stained slide preparations [9].

Synthesis of NCTD

NCTD was synthesised from furan and maleic anhydride via the Diels–Alder reaction. It was kindly provided by Dr Guang-Sheng Wang (Beijing, China).

Cytokine determinations

The concentrations of human IL-1β and tumour necrosis factor (TNF)-α in the serum of healthy donors and in the culture medium of normal human LTBMC or PB-MNC (peripheral blood derived MNC) cultures treated with NCTD or lipopolysaccharide B (LPS; *S. typhosa*) LPS/) (DIFCO, U.S.A.) were determined. Aliquots of supernatants were frozen and stored at −80°C until assayed using Amersham Amerlex-M magnetic separation. The range of detection was 9–289 pg/ml (0.5–16 fmol) for IL-1β and 17–2125 pg/ml (1–125 fmol) for TNF-α. The colony-stimulating activity (CSA) in the culture medium was determined using 10^5 normal human BM-MNC as test cells in 1 ml standard agar gel culture and 20% v/v of serial dilutions of culture supernatant. One CSA unit is defined as the biological activity required to form one GM-CFU on day 14.

Fluorescence-activated cell sorting (FACS) analysis

In 200 μl culture medium, 5 × 10^5 cells were fixed in 70% ethanol at 0°C. The cells were digested with 1000 U RNase A (Sigma) and stained with 1% propidium iodide at 37°C for 30 min [10]. The DNA profiles were determined within 4 h in a Coulter counter and the data were analysed using the Cell Cycle Multi programme.

Macromolecular DNA synthesis

This was determined by measuring [^{3}H]TdR (specific activity 25 Ci/mmol, CEA Orsay, France) incorporation into the acid insoluble material. The mitotic indices (MI per 1000 cells) in control cells and in cells treated with colchicine (0.5 μg/ml), actinomycin-D (Act-D, 0.5 μM), cycloheximide (CHI, 5 μM) or NCTD (0.5–30 μg/ml) were determined on Giemsa-stained cytospin preparations. Cell vitality was determined by Trypan blue dye exclusion.

Electron microscopy

Cell suspensions were centrifuged (200*g*, 10 min), and fixed as a pellet in 2.5% glutaraldehyde–1% osmium tetroxide buffered with PBS (pH 7.2). The cell samples were dehydrated, embedded in Pelco Eponate 12 and analysed by standard procedures.

Statistical analysis

The circadian rhythm of WBC was determined in 25 male, 6-week-old, DBA/2 mice. Means and one standard deviation of the mean (S.D.) were computed for each time point to determine the acrophase and nadir of WBC. The synchronicity of groups of mice along the circadian time scale was verified by measuring body weight, rectal temperature and peripheral WBC in each experiment. The experimental data were compared to time-related controls at the acrophase and at nadir. The colony numbers from duplicate cultures from two to six separate expeiments were analysed. The significance of difference between experimental data was validated using the Student's *t*-test.

RESULTS

Effect of NCTD on leucocytosis and BM cell proliferation in normal mice

Daily i.p. injections of 0.5, 2.0, 5.0 or 12.5 mg/kg NCTD in DBA/2 mice, at the acrophase of peripheral WBC count (6 HALO) (Figure 2a) induced dose- and circadian time-dependent leucocytosis. The greatest stimulation was obtained following three daily injections, both at the acrophase (6 HALO) and at the nadir (15 HALO). The increases in WBC count were 1.2–1.3-fold at acrophase and 1.9–2.2-fold at the nadir over time qualified controls (Figure 2b). The leucocytosis still persisted at nadir after seven injections of 2 or 5 mg/kg NCTD (Figure 2c). The total CFU population per femur was also stimulated, by 17% with the 0.5 and 36% with the 2 mg/kg dose, as measured on day 9 at 6 HALO, at the physiological nadir of CFU populations (not shown). Chronic injection of 0.5, 2 or 5 mg/kg NCTD for 4 weeks had no effect on the peripheral WBC count or on BM cellularity. However, the highest dose, 12.5 mg/kg NCTD, caused acute toxicity and the death of 60% of mice within 24 h of i.p. administration, and all within 48 h.

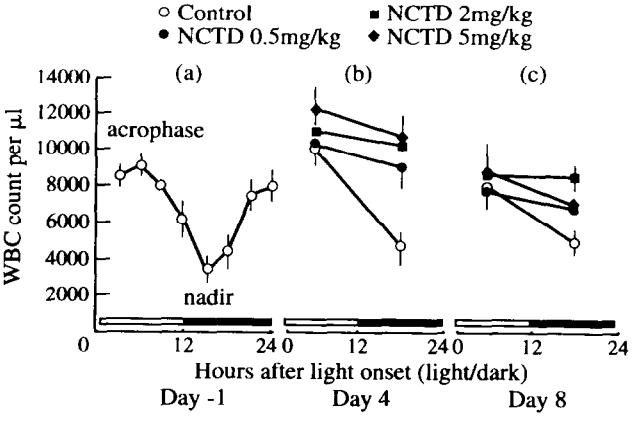

Figure 2. Dose and circadian time-dependent effects of norcantharidin on the peripheral white blood cell (WBC) count in normal, young adult DBA/2 mice. (a) Diurnal rhythm of the WBC level in 24 h in untreated male mice 1 day before norcantharidin treatment. The WBC counts were determined six times on 30 mice synchronised using a 12-h light–12-hr dark schedule. The acrophase was consistently found at 6 h after light onset (HALO), and the nadir at 15 HALO. Groups of six mice were injected intraperitoneally (i.p.) with placebo or with 0.5 mg, 2 mg or 5 mg per kg norcantharidin once a day at the acrophase of WBC count for 7 days. The WBC counts were determined at the acrophase and at nadir on day 4 (b), and on day 8 (c) of treatments. Maximal stimulatory effects were obtained with 5 mg/kg both at the acrophase (1.28-fold the control level; $P < 0.05$) and at nadir (2.21-fold the control level; $P < 0.01$) on day 4. The WBC counts showed persistent stimulation at nadir following seven injections with 2 mg/kg (1.59-fold the control level; $P < 0.01$) or with 5 mg/kg (1.51-fold the control level; $P < 0.05$) norcantharidin.

Effect of NCTD on regenerating BM

Mice were injected with 150 mg/kg 5-FU prior to daily doses of 2 mg/kg NCTD on 5 consecutive days. Table 1 shows that the frequency of HPP-CFC and GM-CFU populations increased significantly over normal BM cells 7 days after 5-FU treatment. This compensatory activation, however, was not significantly altered by NCTD. These results were confirmed in organotypic haematon microcultures using cohorts of randomly selected haematons from control, 5-FU or 5-FU plus NCTD-treated mice. NCTD blocked neither the development of cobblestone area (CA) from single haematons, nor the constitutive production of nucleated cells, nor the GM-CFU populations (Table 1).

Temporal stimulatory effects of NCTD on human BM cultures

Treatment (24 h) with NCTD stimulated both DNA synthesis and increased the frequency of mitotic cells in human liquid BM cultures in a dose-dependent manner. However, the continuous presence of NCTD in these cultures for longer than 3 days resulted in progressive inhibition of DNA synthesis (Figure 3). Short-term preincubation of BM cells in organotypic haematon cultures with NCTD for up to 48 h increased the frequency of both HPP-CFC and GM-CFU populations, as determined in secondary semisolid cultures (data presented together). The continuous presence of NCTD in primary semisolid cultures inhibited both HPP-CFC and GM-CFU populations as a function of dose (Figure 3).

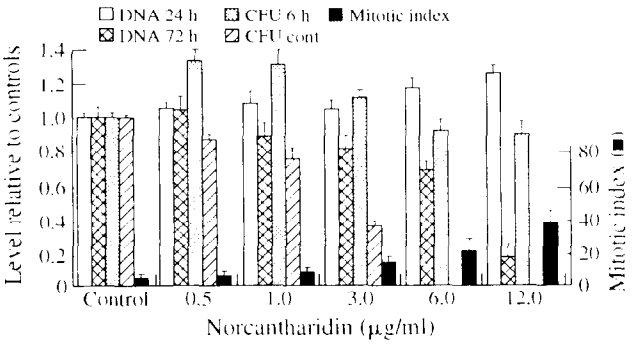

Figure 3. Cellkinetic effects of norcantharidin in human bone marrow-cultures. Norcantharidin had dose- and/or time-dependent biphasic effects on the following parameters: macromolecular DNA synthesis at 24 h and 72 h of treatments; frequency of day 14 granulocyte–macrophage colony forming-unit (GM-CFU) population following a 6-h preincubation and frequency of day 14 GM-CFU population following continuous treatment; and mitotic cells at 24 h of cultures. Data are means of three determinations expressed as relative values to controls; MI, mitotic and/or pseudomitotic cells per 1000 cells. The S.D. values were less than 10% of the means.

Table 1. Effect of norcantharidin on the regenerative ability of haemopoietic progenitor cells or stroma cells in bone marrow following 5-fluorouracil treatment

	Colony-forming unit per 10^5 cells†				Cell production in haematon cultures‡		
Marrow fractions*	HPP-CFC	BFU-E	GM-CFU colony/cluster	Total	Cells × 10^3/well	CFUsec	CAFC
Control							
Buffy coat	0.5 ± 0.6	16 ± 4	43 ± 6/48 ± 5	107 ± 9			
Haematon	2.0 ± 2	27 ± 5	60 ± 7/75 ± 7	164 ± 5	8.9 ± 5	27 ± 17	32/32
5-Fluorouracil							
Buffy coat	1.8 ± 1	62 ± 19	60 ± 8/44 ± 7	168 ± 18			
P§	<0.05	<0.001	<0.01	NS			
Haematon	13.0 ± 4	139 ± 10	122 ± 11/108 ± 10	362 ± 20	7.8 ± 3	199 ± 78	29/32
P	<0.01	<0.001	>0.01	>0.001	NS	<0.001	NS
5-Fluorouracil plus norcantharidin							
Buffy coat	2.1 ± 1	72 ± 35	55 ± 14/37 ± 12	166 ± 27			
P	NS	NS	NS	NS			
Haematon	12.6 ± 5	86 ± 18	91 ± 8/113 ± 16	302 ± 34	6.3 ± 4	220 ± 65	27/32
P	NS	<0.01	NS	<0.05	NS	NS	NS

*The marrow cells were recovered from the femurs of normal, 5-fluorouracil and 5-fluorouracil plus norcantharidin-treated mice 7 days after treatments. The cells from 5 mice in each group were pooled, the buffy coat and the haematon fractions were separated and divided into two parts. One aliquot was dispersed with collagenase/dispase solution to determine the frequency of primary colony-forming unit (CFU) populations; the other was used to establish organotypic liquid haematon cultures. †The frequency of CFU populations was determined in standard semisolid cultures. The data are the means ± S.D. of triplicate cultures. ‡Thirty-two long-term liquid haematon microcultures were established in each group. The constitutively produced non-adherent cells were recovered gently on day 7, the cells counted and the total number of CFU populations per microcultures determined using secondary semisolid cultures (CFUsec). The adherent haematons were overlayered with fresh medium and the development of cobblestone areas with at least five phase dark cells and with active haemopoietic foci was scored on day 28 of cultures (CAFC). §The data for the buffy coat and the haematon fractions from controls and 5-fluorouracil-treated mice were significantly different. Results for 5-fluorouracil-treated mice and 5-fluorouracil plus norcantharidin-treated mice were non-significantly different (except BFU-E). GM-CFU, granulocyte–macrophage CFU; NS, non-significant.

Stimulation of cytokine production by human haematon cultures by NCTD

The secretion of both IL-1β and TNF-α in short-term BM cultures, established with enzymatically dissociated haematons, was stimulated by NCTD. NCTD concentrations above 1 μg/ml induced the production of greater amounts of TNF-α than 5 μg/ml LPS. NCTD plus LPS stimulated TNF-α production in an additive manner. In these cultures, 3 and 6 μg/ml NCTD also significantly ($P < 0.01$) increased the level of biologically detectable CSA (Table 2). Comparable results were obtained with human PB-MNC cultures, where NCTD stimulated both IL-1β and TNF-α production in a dose- and time-dependent manner (Table 2).

Biphasic effects of NCTD on the cell cycle in lower and higher eukaryotic cells

Wild-type *S. cerevisiae* cells were synchronised at the G_1 phase by glucose and nitrogen deprivation at 25°C. The cells were refed and treated with NCTD. The outgrowth of budding daughter cells was quantified 90, 120, 150 and 180 min later. NCTD accelerated the cell cycle transition from the G_1 phase to S and G_2/M phases after 120 min of treatment, while longer treatment inhibited the net cell production as a function of time and dose (Table 3).

Logarithmically growing MRC5V2, HL-60 and K-562 cells were treated with 1, 3, 6 or 12 μg/ml NCTD either alone or in combinations with metabolic inhibitors. NCTD had rapid, dose- and time-dependent effects in these cultures (Table 4). Irreversible mitotic arrest in MRC5V2 and HL-60 cells was induced by 12 μg/ml NCTD. In addition to mitotic arrest, many pyknotic cells were observed in K-562 cell cultures (Figures 5 and 6). NCTD 12 μg/ml caused a greater mitotic arrest than did 0.5 μg/ml colchicine after 24 h of treatment. Act-D and CHI alone decreased the MI and increased the number of pyknotic cells significantly. However, the chromosome paralysing effects of NCTD remained detectable even in the presence of these metabolic inhibitors (Table 4).

FACS analysis of DNA profiles of randomly growing K-562 cells (Figure 4a) indicated that 1 μg/ml NCTD reduced the frequency of G_1 cells and accelerated the G_1/S phase entry (Figure 4b), while 6 μg/ml (Figure 4c) and 12 μg/ml NCTD (Figure 4d) increased the frequency of hypodiploid cells by 14 and 25%, respectively, after 24 h of treatment. NCTD had similar effects in HL-60 cell cultures (Figure 4e). NCTD 1 μg/ml promoted the G_1/S phase transition (Figure 4f) and 6 μg/ml (Figure 4g) and 12 μg/ml NCTD (Figure 4h) concentrations caused a significative G_2/M phase arrest and accumulation of hypodiploid cells.

Cytological and ultrastructural effects of NCTD

Cytological analysis of the MRC5V2, HL-60 and K-562 cell lines revealed that NCTD had no detectable immediate effects on interphase cells, nor did it prevent cytokinesis. However, NCTD (6 or 12 μg/ml) caused several nuclear alterations during the G_2/M and M/G_1 phase transitions. These alterations included premature chromosome condensation, mitotic arrest and the persistance of condensed chromosomes or chromatin aggregates in postmitotic daughter cells (Figures 5a–f). Figure 5e shows typical characteristics of cells undergoing apoptosis in K-562 cell culture. Electron microscopy of K-562 cells showed that NCTD impaired the nuclear organogenesis as it induced characteristic irreversible chromatin condensation and impaired the correct assembly of chromatin material, nuclear membrane and nuclear pore structures (Figures 6a–f).

Table 2. Stimulation of cytokine and haematopoietic growth factor production in human haematon cultures and cultures of peripheral blood derived-mononuclear cells by norcantharidin

Cytokine from†	Culture time (h)	Norcantharidin (μg/ml)* 0	0.5	1.0	3.0	6.0	12.0	LPS plus norcantharidin (μg/ml)* LPS alone	0.5	1.0	3.0
Haematons‡											
IL-1β	24	45	140	135	420	ND	ND	470	480	500	1150
TNFα	24	42	65	85	130	ND	ND	90	140	140	240
CSA	72	105	ND	990	1350	1120	ND	ND	ND	ND	ND
PB-MNC§											
IL-1β											
Donor 1	24	34	ND	36	36	50	105				
Donor 2	24	48	ND	40	47	60	550				
TNFα											
Donor 1	24	20	ND	22	34	100	132				
Donor 2	24	55	ND	52	98	300	1250				

*Parallel cultures were untreated, treated with norcantharidin alone, or with 5 μg/ml LPS alone and with norcantharidin. †Cell-free supernatants were collected after 24 or 72 h of treatment. The concentration of interleukin (IL)-1β and tumour necrosis factor (TNF)-α was determined using the Amersham radioimmunological system. The data are given as fmol/ml. The colony-stimulating activity (CSA) level was determined using serial dilutions of supernatants and normal human bone marrow progenitor cells in semisolid agar gel cultures. The CSA data represent granulocyte–macrophage colony-forming unit (GM-CFU) growth promoting activities, expressed in U/ml. One U is defined as the ability to generate one GM-CFU. ‡Freshly isolated haematons were dissociated in collagenase/dispase, washed three times, and 1 million cells were cultured in 1 ml RPMI-1640 medium containing 10% fetal calf serum. §The peripheral blood-derived mononuclear cell (PB-MNC) fractions were isolated from healthy blood donors by cytopheresis and Ficoll–Hypaque density gradient centrifugation. Mononuclear cells (10^6) were cultured in 1 ml RPMI-1640 medium containing 10% human antibody serum. Cell-free supernatants were collected at 24 and 72 h of culture by centrifugation at 800*g* for 10 min. ND, not determined.

Table 3. Short-term cell cycle promoting and long-term growth inhibitory effects of norcantharidin in synchronised Saccharomyces cerevisiae *cultures following refeeding*

Treatment*	Cell cycle distribution at 120 min (%)† G1	Early S	Late S	G2	M	Mass production (cell × 10^5/ml)‡ 6 h	10 h
Control§	62.4	25.6	8.6	1.8	1.6	20.3 ± 0.2	32.2 ± 0.4
Norcantharidin (μg/ml)							
36	52.8	22.4	16.6	4.8	3.6	19.2 ± 0.3	31.5 ± 0.3
108	48.0	14.2	22.4	12.6	3.4	17.8 ± 0.2	27.0 ± 0.3
216	43.6	9.6	21.4	16.8	8.6‖	11.5 ± 0.2	12.5 ± 0.5‖

*Cells were centrifuged and resuspended in complete medium and parallel cultures were treated with norcantharidin; the cell suspensions were immediately placed in a water bath at 25°C. †In control cultures cell budding started following a lag phase of approximately 75 min and the first cell division approximately 180 min following refeeding. The percentage of cells at different stages of the cell cycles was determined by measurement of the length of buds on 1000 cells according to standard criteria 120 min after refeeding. ‡Cell number (× 10^5/ml) was determined after 6 and 10 h of continuous treatment. Data represent the means of three determinations ± S.D. §Cultures of wild type *Saccharomyces cerevisiae* were established by suspension of industrially produced yeast cells at 500 000 cell/ml concentration in Ca, Mg and sucrose-free phosphate-buffered saline at 4°C. ‖Statistical analysis showed highly significant difference ($P < 0.001$) from controls.

Table 4. Effects of norcantharidin and several metabolic inhibitors on the cell cycle of permanent human cell lines

Treatment*	MRC5V2 MI	IF	HL-60 MI	FUS	PYK	K-562 MI	FUS	PYK†
Control	27	973	43	23	0	22	8	10
Norcantharidin	295	705	409	77	0	114	24	162
Colchicine	148	852	284	12	180	110	0	130
Act-D	1	999	6	0	234	6	0	392
Act-D + norcantharidin	82	918	48	10	460	54	0	318
CHI	5	995	18	42	128	4	10	78
CHI + norcantharidin	105	895	56	22	415	92	0	202
TdR	7	993	30	32	44	3	0	20
TdR + norcantharidin	238	762	ND	ND	ND	110	0	398

*Permanent cell lines in the logarithmic growth phase were treated with 12 μg/ml norcantharidin, 0.5 μg/ml colchicine, 0.5 μM actinomycin D (Act-D), 5 μM cycloheximide (CHI), 2.5 mM thymidine (TdR) either alone or in combinations for 24 h. †Cytokine preparations were stained with May–Grünwald and Giemsa stains and differential counts were made on 1000 cells including: MI, mitotic and pseudomitotic cells; IF, interphase cells; FUS, bi- or multinuclear cells and giant cells; PYK, cells with condensed or fragmented pyknotic nucleus. ND, not determined.

DISCUSSION

These studies show that NCTD, a synthetic analogue of cantharidin, may stimulate haemopoiesis and immunity directly and *via* cytokines, and suppress the growth of human leukaemic cells.

Normal mice given daily injections of 2 or 5 mg/kg NCTD developed significant leucocytosis as measured at the acrophase and at the nadir of peripheral WBC. NCTD also increased the net WBC mass and does not merely shift the circadian rhythm of peripheral WBC. This possibility could not be excluded from previous studies [1]. The leucocytosis was the result of BM stimulation, as NCTD increased the BM cellularity and the total CFU populations in normal, but not regenerating mice. NCTD stimulated the duplication of myeloid progenitor cells and the production of cytokines, including IL-1β, TNF-α and CSA in short-term preincubation experiments, using organotypic haematon cultures. The *in vivo* production of these cytokines could mediate, directly [11, 12] or indirectly [13], the leucocytosis stimulatory effect of NCTD. The stimulation of IL-β, TNF-α and CSA by NCTD in human haematon cultures, therefore, provides the molecular basis for understanding the positive, leucocytosis stimulatory effects of NCTD. The *in vivo* leucocytosis elicited by NCTD was transient, lasting 1 week. The WBC count returned to normal following chronic administration, indicating that NCTD alone cannot sustain the elevated production of blood cells.

Previous studies have shown that NCTD stimulates natural killer (NK) and lymphokine-activated killer (LAK) cell activities in normal human PB-MNC cultures [14]. The present data now provide evidence that these activities may be mediated, at least in part, by increased TNF-α secretion. It is always possible, however, that NCTD activates either the cell adhesion molecules involved in target cell recognition, or the perforin system involved in membrane-mediated target cell killing [15], or both. More detailed toxicity studies are needed to establish whether the acute toxicity of NCTD *in vivo* above the 12.5 mg/kg (LD_{50}) dose is due to the elevated TNF-α level. Our preliminary electron microscopy study in mice has shown that chronic treatment with a subtoxic dose (2 mg/kg) of NCTD, which is equivalent to the therapeutic dose of NCTD in humans [1, 17], had no cardiotoxic side-effects.

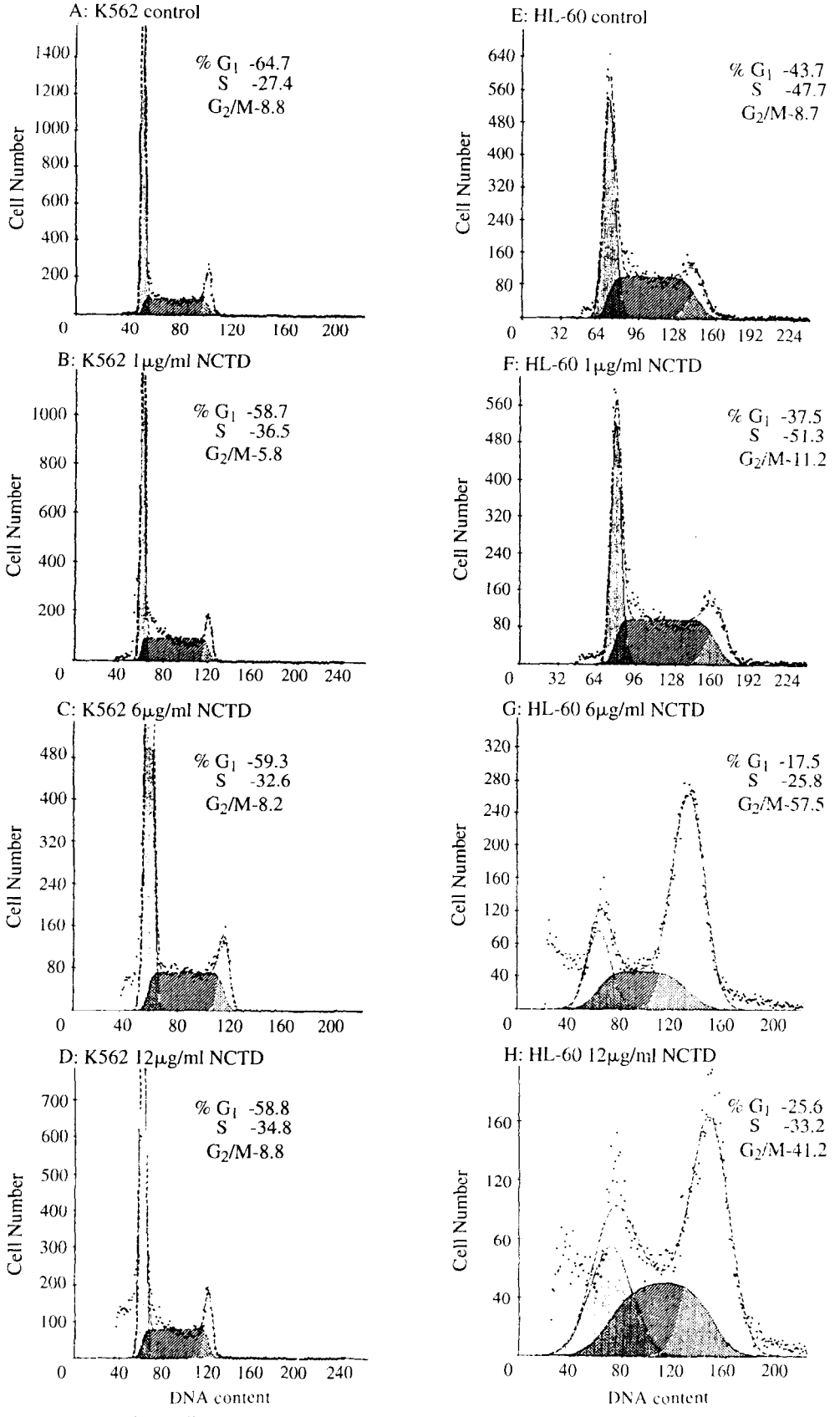

Figure 4. Stimulatory and inhibitory effects of norcantharidin on the cell cycle. Fluorescence-activated cell sorting DNA profiles K-562 cell cultures: (A) randomly growing cells from control culture; (B) cells treated with 1 μg/ml norcantharidin; (C) with 6 μg/ml norcantharidin; (D) with 12 μg/ml norcantharidin for 24 h. HL-60 cell cultures: (E) control cells; (F) cells treated with 1 μg/ml norcantharidin for 24 h. HL-60 cell cultures: (E) control cells; (F) cells treated with 1 μg/ml norcantharidin; (G) with 6 μg/ml norcantharidin and (H) with 12 μg/ml for 24 h. Low concentrations of norcantharidin accelerate the cell cycle progression from the G_1 phase. A high concentration allows the progression of cells from the G_1 phase while it inhibits the cells at the G_2/M phase (HL-60 cells) or inhibits the progression of daughter cells following mitosis (K-562 and HL-60 cells). The daughter cells appear in the hypodiploid (pyknotic/apoptotic) cell gate.

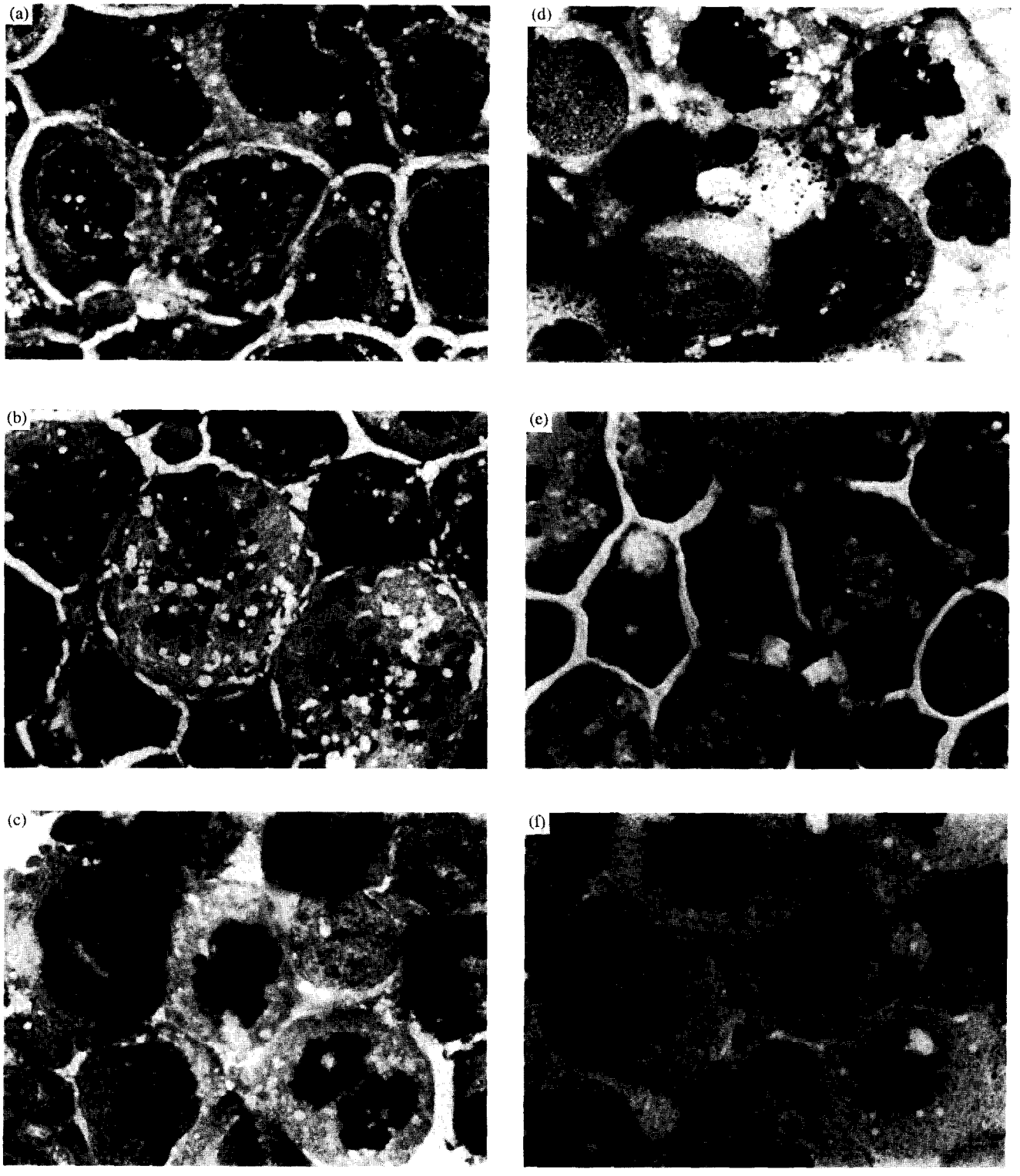

Figure 5. Nuclear alterations induced by incubation with 12 μg/ml norcantharidin for 24 h. HL-60 cell cultures: (a) control culture; note the normal chromosome reorganisation in two daughter cells; (b) impaired and irreversible chromatin and chromosome condensation induced by norcantharidin. K-562 cell cultures: (c) telophase from a control culture; (d) impaired chromatin condensation into gross chromosomes; (e) cells undergoing apoptosis, characterised by nuclear fragmentation from norcantharidin-treated culture. MRC5V2 cell cultures: (f) assymetrical distribution and permanent condensation of chromatin in paired postmitotic daughter cells. Giemsa staining; magnification × 1000.

The question of whether NCTD could affect the cell cycle directly, in the absence of accessory or stromal cells, was also examined. NCTD has been shown to inhibit the growth of HeLa, CHO, HEP-2 and other tumour cell lines [1, 2]. FACS analysis of K-562 cells has indicated that a single 4-μg/ml dose of NCTD induces a marginal 2–2.5-fold increase in G_2/M phases, but no significant decrease in S phase cells up to 5 days of incubation [16]. Our cell pharmacokinetic studies on MRC5V2, HL-60 and K-562 cell lines showed that short incubations with NCTD accelerated the G_1–S phase transition in K-562 cells, in

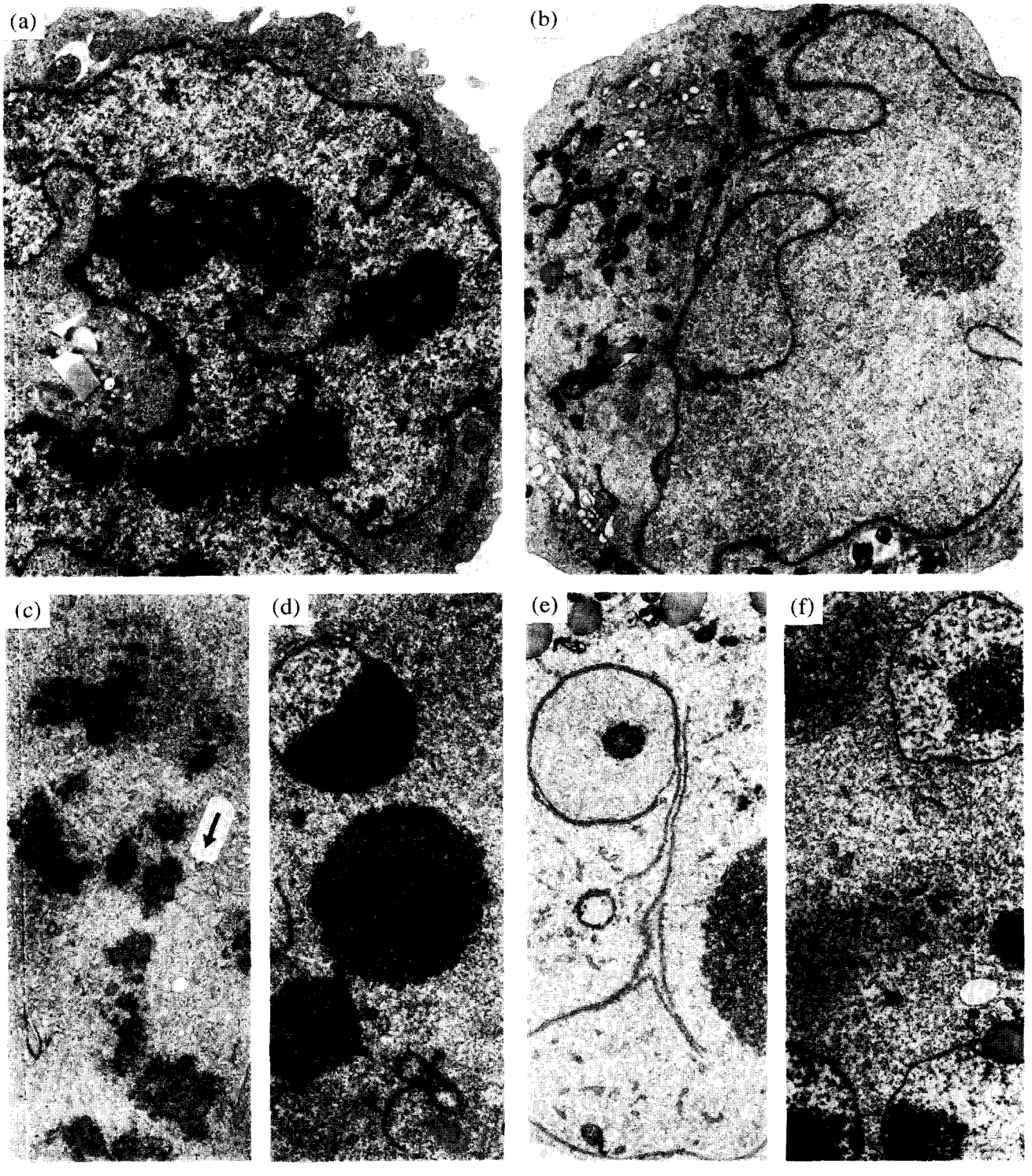

Figure 6. Effect of norcantharidin on the ultrastructure of K-562 cells: (a) interphase cell from a control culture. Cells treated with 12 μg/ml norcantharidin for 24 h: (b) interphase cell with no observable effects on the main cell organelles, (c) persistence of an intact microtubular system and the kinetochor (arrow), and (d–f) irreversibly condensed chromatin surrounded by or lacking the nuclear membrane. Note that the chromatin material remains condensed and the nuclear membrane is either impaired or is dysjuncted from the chromatin. Magnification × 11 000.

agreement with the results obtained in HL-60 cell cultures [17]. We confirmed that NCTD influenced the cell cycle at two distinct points by examining the effects of NCTD on synchronised *S. cerevisiae* cultures. NCTD accelerated cell cycle progression through the G_1 and S phases immediately after treatment, but it inhibited the mass cell production in long-term cultures, as measured two and four generations later. In cultures of human leukaemia cells, NCTD rapidly induced dose-dependent cytological effects at the G_2/M phases, including premature chromatin condensation, prominent M phase arrest, the forma-

tion of bi- or multinuclear cells and irreversible impairment of the reorganisation of nuclear membrane with the chromatin material in postmitotic daughter cells. Cytological and FACS analysis showed that NCTD induced irreversible chromatin condensation at G_2/M phases, which appears in the form of chromosomes in HL-60 cells, and pyknotic nuclei in postmitotic K-562 cells. These results show that NCTD causes programmed cell death.

Recent published information and the present data shed light on the way NCTD acts. Graziano and associates [18] demonstrated that [^{3}H]cantharidin interacts in a saturable and specific manner with a binding site in mouse liver cytosol. This cantharidin binding protein (CBP) was identified recently as serine/threonine-specific PP2A [6]. PP2A is widely distributed throughout the animal and plant kingdoms, and appears to play a critical regulatory role in a number of physiological processes *via* dephosphorylation of a cohort of specific target proteins [4, 19]. Inhibition of PP2A by okadaic acid promotes the cell cycle progression at a start/restriction point, but inhibition of PP2A activity at later stages provokes mitotic defects [3]. A loss-of-function or gain-of-function mutation analysis in yeast and analysis of oocyte maturation in *Xenopus* were used to show that the phosphorylated form of the catalytic p34cdc2 subunit of maturation promoting factor (MPF) forms a preactivated complex with one of the regulatory cyclin subunits. The functional activation of the p34cdc2/cyclin complex requires dephosphorylation of p34cdc2 at the Tyr-15 residue, either by the phosphatase designated p80cdc25 in yeast [20] or by p72 in *Xenopus* [21]. We suggest that the acceleration of G_1–S phase transit in *S. cerevisiae* cultures, in human BM cultures and in permanent human cell lines might be due to activation of the p34cdc/cyclin-A system at the restriction point as a result of neutralisation of PP2A activity by NCTD.

Another cell cycle inhibitory effect of NCTD was identified at the G_2/M and M/G_1 phase transitions. NCTD induced the disappearance of the nuclear membrane and the premature and irreversible condensation of chromatin. NCTD did not inhibit cytodieresis, but it abrogated the redispersion of chromosomes, and the neogenesis of nuclear membrane and nuclear pore complexes in postmitotic daughter cells. Thus, NCTD may have effects on the microtubular system similarly to those of okadaic acid [22]. These mitotic defects can also be understood from the inhibition of PP2A activity by NCTD and the subsequent activation of p34cdc2/cyclin-B complex [23]. Cyclin-B accumulates in the perinuclear region of the cytoplasm during G_2 in HeLa cells, enters the nucleus at prophase and then accumulates at the spindle cap [24]. The translocation of the cyclin moiety could ensure the specific assemblage of the catalytic p34cdc2 with critical substrates [25] such as components of the nuclear membrane [26] and the residual matrix proteins, histone H1 [27], major nucleolar proteins [28] and contractile claret proteins [24, 29]. Other studies, using deletion and point mutant strains for two PP2A genes ppa1+ and ppa2+ in *Saccharomyces pombe*, have shown that decreased PP2A (and PP1) activity causes mitotic defects, premature mitosis and chromosome non-disjunction [29].

These studies indicate that PP2A negatively regulates p34cdc2 kinase activity under physiological conditions. Consequently, the specific inhibition of PP2A may result in activation of the p34cdc2 kinase system. Our results support the idea that NCTD stimulates or inhibits distinct stages of the eukaryotic cell cycle via PP2A by interacting with the p34cdc2/cyclin M-phase kinases [21, 24]. These kinases have biphasic, periodic effects in most of the eukaryotic cells studied.

Recent clinical trials with NCTD as a monotherapeutic agent indicate that it is effective against primary hepatoma and against oesophageal, gastric and cardiac carcinomas [1]. NCTD increased the mean survival time of 285 reported cases with primary hepatoma from 4.7 to 11.1 months, and the 1-year survival rate from 17 to 30%, as compared to 102 patients treated with conventional chemotherapy (5-FU, hydroxycamptotherine, vincristine, thiophosphoramide and mitomycin) [1]. These studies suggest that NCTD is a potential antineoplastic agent, and it may be useful for developing new therapeutical regimens for the treatment of solid tumours and perhaps some haemopoietic malignancies [30].

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Epidermal Growth Factor-induced Protection of Tumour Cell Susceptibility to Cytolysis

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Using radiobinding, transfection and colorimetric assays, the biological significance of epidermal growth factor (EGF) and its receptor on established human tumour cell lines was investigated. The intensity of class I major histocompatibility antigen (MHC) and EGF receptor (EGFR) expression on 20 tumour cell lines was investigated and showed no direct correlation (coefficient of correlation $r = 0.43$ and $P = 0.06$). Furthermore, transfection of the β2-microglobulin gene into a class I negative bladder tumour cell line, resulting in the re-expression of fully assembled cell surface class I antigens, did not result in alteration of EGFR expression. However, there was an inverse correlation between the intensity of EGFR expression and the stimulatory response of cells to exogenously added EGF. The per cent inhibitions of cell proliferation by EGF at 100 ng/ml for A431 (highest EGFR expressor) and Scaber (lowest EGFR expressor) were 37 and −7%, respectively. The results also showed that cell lines isolated from testis tumours positive for epithelial markers (using pan keratin antibody LP34 as an epithelial marker), expressed significantly lower EGFR levels than cell lines from bladder tumours. The expression of EGFR receptor was not modulated by interferons (IFN-α and -γ and only a minor effect with IFN-β) or active supernatant containing a mixture of cytokines. Whilst the pretreatment of tumour cells with IFNs resulted in a significant increase in the susceptibility of tumour cells to interleukin-2-activated peripheral blood mononuclear cells, EGF treatment resulted in their protection. Thus, the per cent killing at an effector:target ratio of 20:1 for untreated cells and EGF (100 ng/ml), IFN-α (1000 U/ml), -β (2000 U/ml) and -γ (100 U/ml) were 53%, 33% ($P = 0.004$), 64% ($P = 0.004$), 69% ($P = 0.001$) and 66% ($P = 0.001$), respectively. These results indicate the complex interactions between EGF and EGFR and their relevance in modifying tumour cell behaviour. The hypothesis that the resistance to cytolysis of tumour cells induced by EGF stimulation may be a factor in the accelerated tumour growth seen in patients after traumatic tissue damage is discussed.

Key words: epidermal growth factor, epidermal growth factor receptor, major histocompatability complex, transfection, cytokine
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