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Nitrosoureas Lomustine, Carmustine and Fotemustine Induced Hepatotoxic Perturbations in Rats: Biochemical, Morphological and Flow Cytometry Studies

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Chloroethylnitrosoureas are reactive compounds that are highly effective against malignant neoplasms in humans and animals. The most widely used nitrosoureas, lomustine and carmustine, are known to be hepatotoxic and to induce pericholangitis and intrahepatic cholestasis, which in the long term lead to cholangiolysis and biliary cirrhosis. However, the nitrosourea fotemustine has proved to be non-hepatotoxic at 20 mg/kg and 50 mg/kg. We have studied the effect of these three nitrosoureas on the cytotoxicity and cellular kinetics of rat liver cells. Lomustine and carmustine modify the proliferation index of liver cells *in vivo*: flow cytofluorometry showed that DNA cell distribution is quite similar for lomustine and carmustine, with subsequent accumulation of cells in G_2 + M phase. 3 months later regressive morphological and cell cycle perturbations are noted for the lower dose of lomustine and carmustine. The most severe lesions are noted with lomustine (50 mg/kg). Fotemustine is not hepatotoxic and preferentially induces S phase perturbations. The more toxic nitrosoureas, lomustine and carmustine, induce comparable hepatocyte cell cycle alterations which differ from those induced by the less hepatotoxic nitrosourea fotemustine.
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INTRODUCTION

CHLOROETHYLNITROSOUREAS are antitumour agents active in the treatment of experimental malignant tumours such as lymphomas, melanomas, lung and brain tumours and intracranial and systemic L1210 leukaemia [1, 2].

The currently used carmustine and lomustine easily cross the blood–brain barrier and are the most effective agents available for the treatment of primary brain tumours [3]. New derivatives of nitrosoureas (such as fotemustine), a new nitrosourea consisting of an aminophosphonic acid group grafted into a nitrosourea radical, have been synthesised in an effort to achieve better cellular penetration and passage through blood–brain barrier.

The major side-effects of these compounds are cumulative bone marrow, liver, pulmonary and renal toxicity [4–6]. The hepatotoxicity of carmustine and lomustine has been well studied [7, 8]. Recent laboratory studies showed that carmustine induces hepatic lesions similar to those of lomustine while the new nitrosourea fotemustine is not hepatotoxic [9].

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We have previously reported that lomustine induces pericholangitis and intrahepatic cholestasis with microtubular abnormalities. In a first phase, transaminases increases and inflammation of the portal tracts occurs while in a second phase a rise in transaminases is noted with marked dilation of bile canaliculi and numerous filament bundles dispersed in the cell cytoplasm [10].

Brodie *et al* [11] showed that some nitrosoureas (lomustine, carmustine, semustine) interact with microtubules; nitrosoureas which form isocyanates are particularly able to inhibit the polymerisation of purified brain tubulin in a dose-dependent manner. Indeed, nitrosoureas affect cell cycle progression in G_2 phase [12]. It was shown in the human lymphoma cell line T_1 that cell cycle effects were different between nitrosoureas (carmustine, lomustine, semustine) and might contribute to the anticancer activity of the drugs [13].

Because the hepatotoxic effects of nitrosoureas imply microtubular abnormalities, the purpose of this study was to compare the impact of the administration of three nitrosoureas, lomustine, carmustine and fotemustine, on the liver cell cycle in rats and to correlate biochemical, morphological and cytofluorometric studies.

MATERIALS AND METHODS

Animals

Female Wistar rats weighing approximately 200 g with free access to food and water throughout the study were given a single dose of lomustine, carmustine or fotemustine.

Drugs

Lomustine was supplied by Roger Bellon (France), carmustine by Bristol Myers (France) and fotemustine by Servier (France). Lomustine was prepared in 1% carboxymethylcellulose solution. Animals were given a final volume of 1 ml by gastric intubation. Carmustine and fotemustine were dissolved in 95% ethanol (50 mg/l), freshly diluted in glucose 5% solution and injected intravenously. Control animals were injected intravenously with 4 ml/kg body weight of glucose 5% solution.

Treatment schedules

A total of 192 animals were divided into 8 groups of 8 animals. Animals received a single dose of 20 or 50 mg/kg lomustine, carmustine or fotemustine and were killed 30, 60 or 90 days later. Groups of respective controls were killed in the same manner.

Biochemical studies

Total plasma bilirubin, alkaline phosphatase and alanine aminotransferase (ALT) activities were assayed colorimetrically as previously described [10]. The statistical significance of results was evaluated by analysis of variance and complementary analysis of Dunnett's test for the comparison of means.

Morphological studies

Histological study was performed on each animal. The animals were perfused with 100 mmol/l phosphate buffered saline (PBS) (pH 7.6). Liver specimens were fixed in formaldehyde 10% for 24 h and embedded in paraffin, and 5 μm sections were stained with haematoxylin-eosin, periodic acid-Schiff and Masson trichromic stain.

For electron microscopy, 1 mm^3 liver blocks were treated as previously described [10]. Briefly, liver tissues were fixed in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide, then embedded in epoxy resin, stained with uranyl acetate and lead citrate, and examined with a Philips CM 10 transmission electron microscopy.

Flow cytometric study

Sample preparation. Animals were perfused with 100 mmol/l phosphate buffer pH 7.6. 1–2 mm^3 liver samples were immediately removed and placed in citrate buffer containing 250 mmol/l sucrose, 40 mmol/l trisodium citrate, 5% dimethylsulphoxide (DMSO) pH 7.6, according to the procedure of Vindelov *et al.* [14], then stored at −80°C.

Before analysis, the samples were thawed rapidly in a water-bath at 37°C. Blocks of tissues were mechanically disaggregated with scissors in PBS buffer and the single cell suspension was then filtered through 48 μm pore nylon gauze. Cell count was performed to obtain 10^4 to 10^6 cells/ml.

This suspension was mixed with chicken red blood cells (CRBC) which were used as internal standard as described by Vindelov *et al.* [14]. The concentration was adjusted to obtain a final ratio CRBC/liver cells of 1/10. An aliquot of 200 μl of these nuclear suspension samples was added to 500 μl of a staining solution including RNase, propidium iodide and non-ionic detergent Nonidet P40 for 10 min at 4°C as described by Vindelov [15].

Flow cytometric analysis. The DNA content of stained single cell suspension was measured using an EPICS C flow cytometer (Coultronics) with the 488 nm band of an ion argon laser as the excitation source.

Table 1. Serum ALT in nitrosourea-treated rats

ALT (U/l)	Day 30	Day 60	Day 90
Control	49.5 (2.4)	34.6 (1.9)	46.6 (4.9)
Lomustine			
20 mg/kg	71.8 (5.1)†	45.5 (3.8)	68.8 (9.2)*
50 mg/kg	88.0 (6.6)†	53.8 (7.1)*	43.4 (2.8)
Control	42.9 (2.4)	25.5 (1.7)	38.3 (2.2)
Carmustine			
20 mg/kg	65.9 (4.8)	52.0 (4.4)†	63.7 (4.2)*
50 mg/kg	72.2 (4.4)†	55.7 (5.9)†	71.9 (11.5)†
Fotemustine			
20 mg/kg	48.5 (3.5)	34.4 (1.4)	51.4 (5.6)
50 mg/kg	50.2 (3.3)	41.0 (3.7)	34.0 (2.8)

Rats were killed 30, 60 or 90 days following administration.
Values are the means (S.D.) of 8 independent experiments.
*$P < 0.05$ vs. controls; $P < 0.01$ vs. controls (Dunnett's test).

The cytofluorograph was adjusted to maximal resolution with polystyrene microspheres (Odam-Brucker, Strasbourg, France) having a coefficient of variation (CV) less than 1.5% and with hyperplastic lymph-nodes with a ratio 4c/2c = 1.9 to 2.0. For each animal, 10^4–10^5 particles were analysed. The CV of diploid peaks ranged from 2.2 to 17.5.

The peaks with a CV > 7% were excluded from this study. For each histogram, the diploid DNA content of rat liver cells was determined by the CRBC ratio: the mean of the G_0G_1 peak divided by the mean (S.D.) of CRBC peak was 2.76 (0.03). The calculation of the cell cycle distribution was performed using the CYTOLOGIC software (Coultronics) equipped with four mathematical models: Baisch's I model, Baisch's II model, Dean's model and Fried's model [16–18]. For any model, the analysis of S and G_2 + M phases was not always possible because of overlapping peaks, particularly when S phases were very high.

For each histogram, the parameters studied were G_0G_1 phase (2c DNA content), the index of proliferation (IP_1) expressed as S + (G_2 + M) and representing the percentage going to cycle, and the S phase (2c < DNA < 4c) and G_2 + M(4c DNA content), also separated in order to determine the specific impact of the tested drugs.

The increase in the percentage of cells in G_2 + M phase was interpreted as an accumulation of tetraploid cells which showed an inhibition of cytodieresis. Nevertheless, these cells were able to divide by endoploidy. This phenomenon was quantified by the percentage of cells with DNA content comprised between 4c and 8c (IP_2).

Statistical analysis. Results were expressed as mean (S.D.). Analysis of variance was performed between groups using the non-parametric rank test of Kruskall–Wallis for each interval. Three groups were compared after oral administration (gastric intubation for controls, lomustine 20 or 50 mg/kg), and 5 groups after intravenous injection (controls, carmustine 20 or 50 mg/kg, fotemustine 20 or 50 mg/kg). Complementary analysis was made for groups in which P was less than 0.05 [19].

RESULTS

Specific liver function tests

Hepatic transaminases (*Table* 1). A single dose of 20 mg/kg lomustine induced a biphasic rise in ALT 30 and 90 days following administration. The higher dose of 50 mg/kg lomustine

Table 2. Serum alkaline phosphatase in nitrosourea-treated rats

Alkaline phosphatase (U/l)	Day 30	Day 60	Day 90
Control	34.0 (1.7)	34.8 (3.2)	26.7 (3.5)
Lomustine			
20 mg/kg	36.8 (1.9)	32.4 (3.3)	30.6 (3.7)
50 mg/kg	100.1 (5.3)†	77.0 (1.5)†	112.6 (11.0)†
Control	32.2 (2.5)	40.6 (2.5)	30.0 (2.9)
Carmustine			
20 mg/kg	50.5 (5.9)*	34.0 (7.0)	22.3 (2.9)
50 mg/kg	65.2 (6.0)†	32.6 (2.7)	32.8 (2.2)
Fotemustine			
20 mg/kg	34.0 (2.9)	28.3 (2.8)	24.5 (3.1)
50 mg/kg	33.0 (2.7)	33.5 (1.8)	24.8 (1.3)

Values are the means (S.E.) of 8 independent experiments.
*$P < 0.05$, †$P < 0.01$ vs. controls (Dunnett's test).

Table 3. Serum total bilirubin in nitrosourea-treated rats

Total bilirubin (mg/l)	Day 30	Day 60	Day 90
Control	1.7 (0.15)	2.7 (0.3)	1.4 (0.1)
Lomustine			
20 mg/kg	3.0 (0.2)	2.4 (0.2)	2.5 (0.25)
50 mg/kg	8.2 (0.6)†	6.6 (1.0)†	32.5 (5.2)†
Control	2.8 (0.15)	1.7 (0.12)	2.3 (0.3)
Carmustine			
20 mg/kg	2.2 (0.6)	2.5 (0.13)†	2.5 (0.2)
50 mg/kg	13.5 (4.4)†	2.2 (0.14)	3.1 (0.25)
Fotemustine			
20 mg/kg	1.2 (0.3)	2.0 (0.13)	2.4 (0.15)
50 mg/kg	2.6 (0.12)	1.7 (0.14)	1.8 (0.16)

Values are the means (S.E.) of 8 independent experiments.
†$P < 0.01$ vs. controls (Dunnett's test).

induced a marked rise in ALT levels on day 30 [88.0 (6.6) U/l, $P < 0.01$], which decreased on day 60 [53.8 (7.1) U/l, $P < 0.05$] and returned to normal on day 90 [43.4 (2.8) U/l]. A single dose of 20 or 50 mg/kg carmustine induced significant rises in transaminases at the various intervals, with a similar slight decrease in ALT on day 60 for both the doses. The administration of 20 or 50 mg/kg fotemustine induced no change in the transaminase activity whatever the interval.

Serum alkaline phosphatase (*Table* 2). No change in alkaline phosphatase levels was observed with a single dose of 20 mg/kg lomustine. The dose of 50 mg/kg lomustine induced a marked increase in alkaline phosphatase on day 30 [100.1 (5.3) U/l, $P < 0.01$], on day 60 [77.0 (1.5) U/l, $P < 0.01$] and on day 90 [112.6 (11.0) U/l, $P < 0.01$] compared with the mean value of control [34.8 (3.2) U/l].

The doses of 20 and 50 mg/kg carmustine induced a lesser increase in alkaline phosphatase, only on day 30 [50.5 (5.9) U/l, $P < 0.05$ for 20 mg/kg and 65.2 (6.0) U/l, $P < 0.01$ for 50 mg/kg]. This increase was less than with lomustine. The alkaline phosphatase activities did not vary whatever the dose or interval after treatment with fotemustine.

Serum total bilirubin (*Table* 3). The single dose of 20 mg/kg lomustine did not change the serum bilirubin levels in treated rats. The dose of 50 mg/kg induced a rise in total bilirubin on day 30 [8.2 (0.6) ml/l, $P < 0.01$] and on day 60 [6.6 (1.0) mg/l, $P < 0.01$] compared with controls [1.7 (0.15) mg/l]. High bilirubinemia was noted on day 90 [32.5 (5.2) mg/l, $P < 0.01$ vs. 1.4 (0.1) mg/l in controls]. The 20 mg/kg dose of carmustine induced a slight increase in serum total bilirubin only on day 60. With the dose of 50 mg/kg, total bilirubin showed a more pronounced rise of day 30 [13.5 (4.4) mg/l, $P < 0.01$ vs. 2.8 (0.15) mg/l in controls] and returned to normal values on days 60 and 90. Treatments with 20 or 50 mg/kg fotemustine did not affect the levels of total bilirubin in rats.

Morphological studies

Hepatic lesions induced by nitrosoureas were considered in three categories: parenchymal, mesenchymal and biliary.

Parenchymal lesions consisted of coagulation necrosis which was paucicellular or focal. Mesenchymal lesions were oedema and fibrosis, localised to the portal tracts or extending into the liver parenchyma to form a nodular pattern. In the latter case, extensive fibrosis was always associated with ductular proliferation.

The intensity of biliary lesions was variable and involved the biliary epithelium and periductular area. According to the intensity of alterations, minor lesions were characterised by bile duct dilation surrounded by an irregular epithelium leading to sclerosing cholangitis aspects with reduced lumens, lined by fibrous thickening of the walls. The bile duct epithelium was strongly dystrophic or had disappeared. More severe lesions were first characterised by epithelial destruction accompanied by inflammatory polymorph infiltration of the biliary duct wall (acute cholangitis), then giving rise to cholangiolysis lesions in which the lumen had totally disappeared, replaced by a dense fibrous tissue rich in fibroblasts, then by dense fibrous acellular scarring.

No parenchymal, biliary or portal lesions were observed in control animals whatever the interval (Fig. 1A).

Lomustine treatment. For a dose of 20 mg/kg, on day 30, 8 animals showed minor biliary lesions within 2 cases, discrete periportal fibrosis. On day 60, 6 animals showed mild lesions. 2 animals had sclerosing cholangitis. On day 90, lesions were always stable for each animal and consisted of reduced lumens of the bile duct with slight inflammatory pericanalar fibrosis.

For a dose of 50 mg/kg, on day 30, minor alterations of bile ducts were accompanied by foci of hepatocytic necrosis. On day 60, four animals showed sclerosing cholangitis lesions, which in one were subcapsular foci of adenomatous transformation (Fig. 1F). On day 90, 6 animals showed severe lesions with alterations of the hepatic parenchyma: the portal tract was enlarged and fibrous. Fibrosis was slightly extended and tended to form nodular areas of hepatic parenchyma separated by thin bands of connective tissue (Fig. 1E). Ductular proliferation was also noted and seemed related to hepatocyte dedifferenciation. Two animals showed lesions of cholangiolysis.

Carmustine treatment. For a dose of 20 mg/kg, on day 30, 6 animals showed severe lesions. The biliary epithelium was strongly dystrophic and surrounded by dense pericanalar fibrosis. 2 animals presented cholangiolysis (Fig. 1D). On day 60, discrete biliary alterations coexisted with an accentuation of

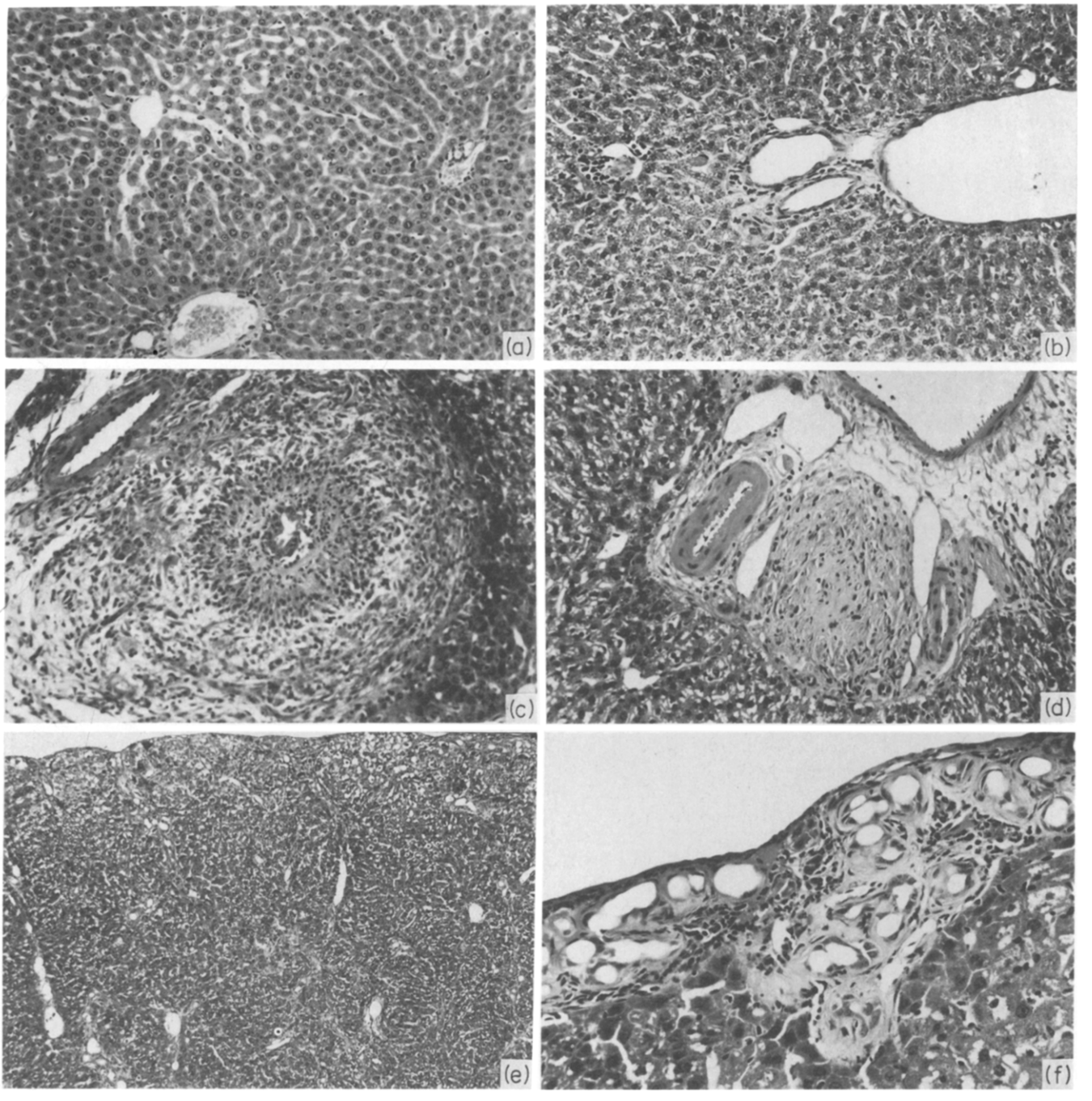

Fig. 1. Mild to severe nitrosourea-induced lesions. (a) Control rats. Normal hepatic parenchyma (×250). (b) 50 mg/kg fotemustine-treated rats on day 30. Slight dilation of bile ducts (×250). (c) 50 mg/kg carmustine-treated rats on day 30. Acute cholangitis with necrotic epithelial cells in the lumen and polymorph granulomas infiltrate, thickening the wall (×250). (d) 20 mg/kg carmustine-treated rats on day 30. The biliary lumen has totally disappeared: the bile duct is replaced by a dense fibrous scar with few inflammatory infiltrates (×250). (e) 50 mg/kg lomustine-treated rats on day 90. Architectural perturbations of hepatic parenchyma outlining a nodular pattern (×25). (f) 50 mg/kg lomustine-treated rats on day 60. Adenomatous biliary changes (×400).

the network of connective tissue in periportal areas. On day 90, lesions were regressing: 5 animals showed minor hepatocytic lesions.

For a dose of 50 mg/kg, on day 30, 3 animals showed minor lesions, 4 had sclerosing cholangitis and 1 showed altered bile ducts with polymorphic granulomatous infiltration (Fig. 1C). On day 60, 5 animals had minor lesions and 4 had lesions similar to those previously described for the dose of 20 mg/kg. 1 animal had destroyed biliary ducts. On day 90, 2 animals showed reversible lesions and 6 had discrete or unchanged scarring lesions.

Fotemustine treatment. No lesions were observed with fotemustine, both for 20 and 50 mg/kg and for each length of treatment. The liver was normal and non-significant lesions were characterised by slight dilation of bileducts, discrete pericanalar fibrosis or inflammatory infiltrate surrounding portal areas (Fig. 1B).

Ultrastructural study was conducted in rats treated with a single dose of 50 mg/kg of lomustine, carmustine or fotemustine. Both lomustine and carmustine induced lesions localised to bile canaliculi, showing dilation and reduction of canalicular microvilli. These alterations have been described previously [10]. There was no alteration of liver cells with fotemustine.

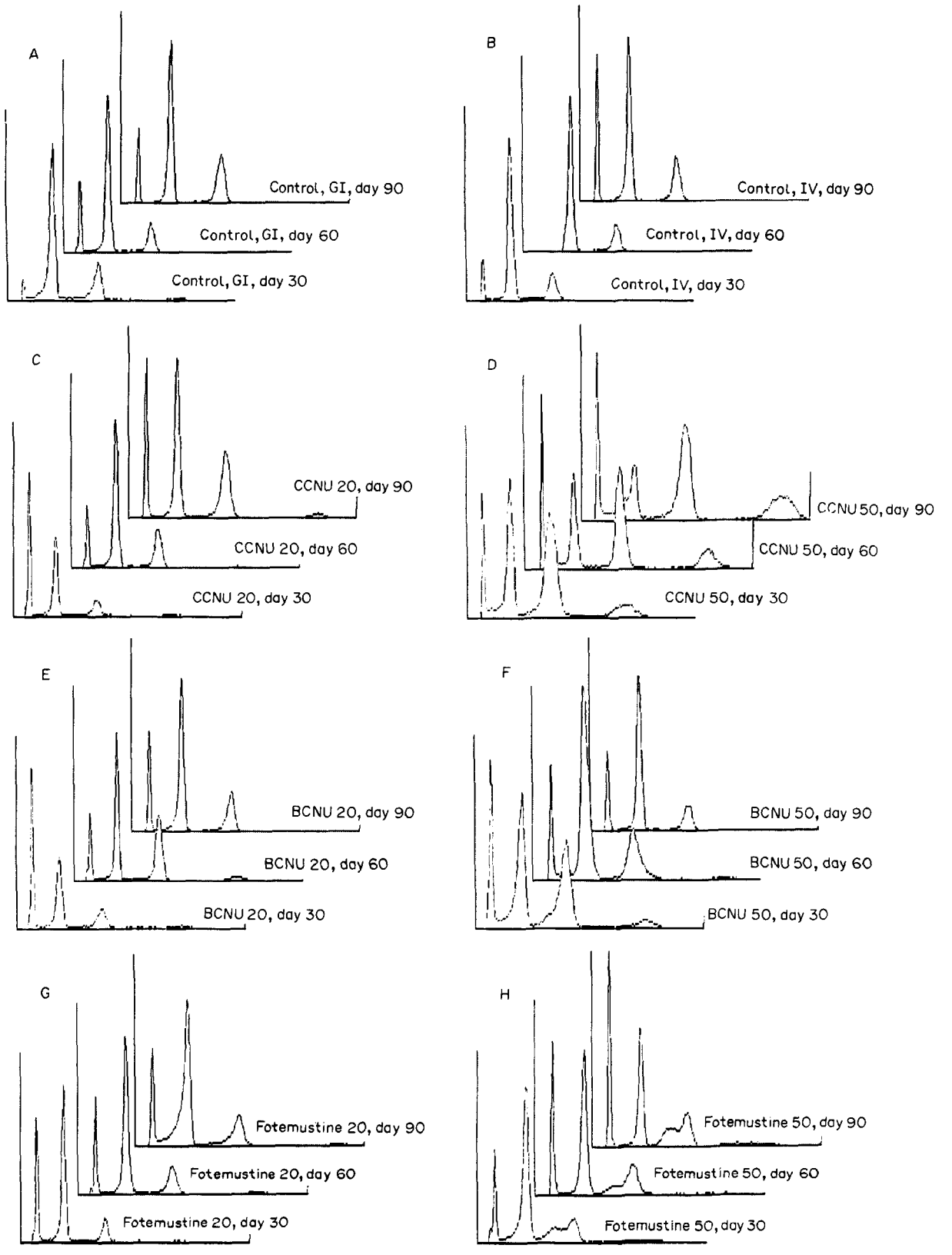

Fig. 2. DNA distribution of liver cells in controls and treated rats with a single dose of lomustine, carmustine and fotemustine. Flow cytometric analysis was performed using propidium iodide. DNA histograms were obtained after an interval of 30, 60 or 90 days for each molecule. (A) control, gastric intubation (GI); (B) control, intravenous injection (IV); (C) lomustine 20 mg/kg; (D) lomustine 50 mg/kg; (E) carmustine 20 mg/kg; (F) lomustine 50 mg/kg; (G) fotemustine 20 mg/kg; (H) fotemustine 50 mg/kg.

Flow cytofluorimetric analysis

The analyses of cell cycle distribution of liver cells from rats treated with lomustine, carmustine and fotemustine are presented in Tables 4, 5 and 6, respectively. DNA histograms are depicted in Fig. 2. Comparative index of proliferation (IP_1) is shown in Fig. 3.

Controls. Figs 2A, 2B and 3 and Tables 4–6. The percentage of cells in the different phases of cycle (G_0G_1, S, G_2 + M) and the index of proliferation were similar for each control. The percentage of G_0G_1 cells varied from 61.1 to 72.2%, the CV was comprised between 3.6 and 4.8% and the index of proliferation (IP_1) between 28 and 38.5%.

Lomustine treatment. (Figs 1C, 1D and 3A, Table 4). Whatever the dose or time of death, similar perturbations in cell cycle were observed. These alterations concerned preferentially the G_2 + M phase which was dramatically increased and significantly different from controls with the dose of 50 mg/kg for each treatment length ($P < 0.01$) (Fig. 3A). The G_2 + M arrest was considered as blockage of mitosis and inhibition of cytodieresis. This alteration first induced a great decrease in G_0G_1 phase of the cell cycle at days 30, 60 and at day 90 was accompanied by a significant accumulation of cells in S phase.

Some tetraploid cells divided towards the octaploid cycle and represented an endoploidy phenomenon. These alterations were progressive, and percentages of octoploid cells were always higher with longer treatment. At day 90, almost 50% of tetraploid cells were engaged in endoploidy with CCNU 50 mg/kg. In contrast, with the dose of 20 mg/kg, the alterations seemed more extensive earlier.

Carmustine treatment. (Figs 2E, 2F and 3B, Table 5). A similar effect was observed on the G_2 + M phase. However, none of the alterations was significantly different from controls at any treatment time. With the high dose, the first significant effect was noted at day 30 on the G_2 + M compartment, with a parallel reduction of the G_0G_1 compartment. At later times, further perturbations developed with a significant accumulation of cells in S phase ($P < 0.05$). At day 90, these alterations seemed to diminish and the cell cycle of treated rats was not significantly different from that of controls (Fig. 2F). Endoploidy was commonly observed at each interval, but reached a maximum at day 60. A regressive phenomenon was observed with IP_1 (Fig. 3B).

Fotemustine treatment. (Figs 2G, 2H and 3C, Table 6). Cell cycle perturbations after fotemustine were different from those induced by lomustine and carmustine and were particularly prominent with the 50 mg/kg dose. The major disturbance was seen on S phase cells. This modification persisted throughout the study and particularly affected the late S phase. These modifications were so marked that for some animals, cell cycle estimation was not possible and did not allow statistical analysis for this group.

It is noteworthy that a minor effect on mitosis was observed at days 60 and 90, although the significant S phase perturbation persisted. At each time, the index of proliferation was not strongly modified except for fotemustine 50 mg/kg at day 60 (Fig. 3C).

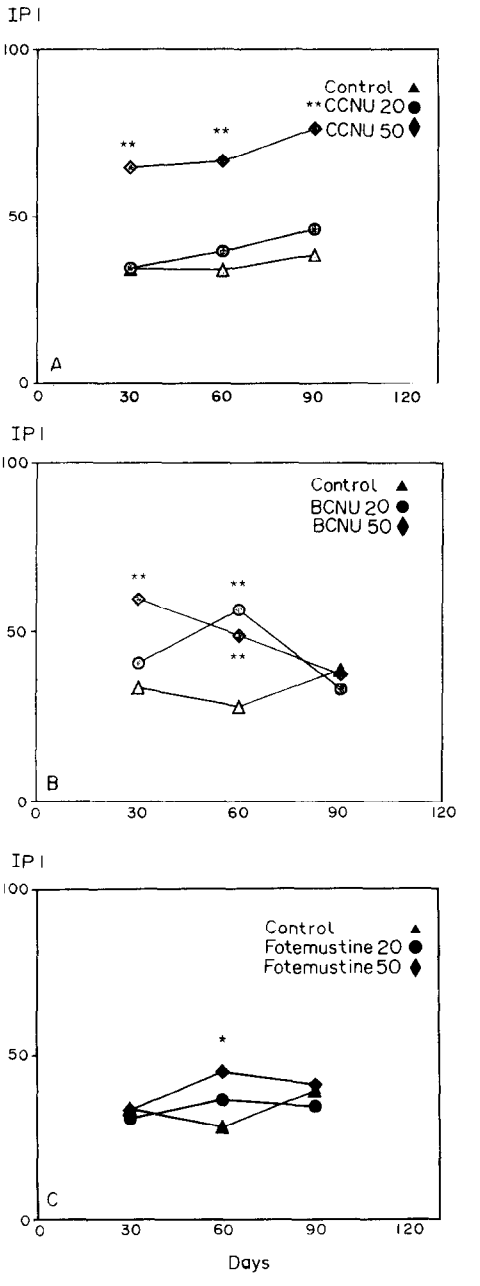

Fig. 3. Comparison of index of proliferation (IP_1) in rats treated with a single 20 mg/kg or 50 mg/kg dose of nitrosoureas. (A) lomustine; (B) carmustine; (C) fotemustine. Means (S.D.) of 8 independent experiments (*$P < 0.05$; **$P < 0.01$, Kruskall–Wallis' test).

DISCUSSION

Nitrosoureas are chemotherapeutic agents that have toxic side-effects, such as myelosuppression and gastrointestinal, hepatic, renal and pulmonary toxicities, that limit their clinical usefulness [4–7]. However, our studies showed differences in the hepatotoxicities of the three nitrosoureas. In this study, we attempted to determine whether there is a relationship between the hepatic alterations and the potent cell cycle modifications following nitrosourea administration. The biochemical and morphological studies are similar to previous reports and confirmed that lomustine induced pericholangitis and intrahepatic cholestasis with microtubular abnormalities, with a long-term evolution of hepatic alterations to cholangiolysis and biliary lesions [10]; carmustine induced similar lesions after a single administration. Furthermore, focal hepatocellular necrosis occurred following repeated doses but as no long-term evolution to cirrhosis was

Table 4. Effect of lomustine on cell cycle progression capacity of rat hepatocytes

	% of cells in					
	G_0G_1	S	G_2 + M	G_2 + M(8c)	IP_1	IP_2
Day 30						
Control	67.1	20.8	12.1	—	34.3	—
(n = 7)	(6.0)	(9.0)	(4.9)		(6.2)	
Lomustine						
20 mg/kg	65.4	19.3	15.1	8.3	34.6	29.2
(n = 8)	(5.4)	(4.8)	(4.8)	(5.1)	(5.3)	(9.3)
50 mg/kg	35.4	28.9	35.7	14.4	64.6	31.1
(n = 8)	(6.1)†	(10.3)	(12.4)†	(4.6)	(6.1)†	(5.2)
Day 60						
Control	66.0	14.4	19.6	—	34.0	—
(n = 8)	(2.6)	(2.9)	(3.0)		(12.6)	
Lomustine						
20 mg/kg	60.5	15.3	24.2	3.0	39.5	15.6
(n = 8)	(18.0)	(5.6)	(6.6)	(2.0)	(10.8)	(3.0)
50 mg/kg	33.4	18.8	47.8	18.9	66.6	35.9
(n = 8)	(9.4)†	(9.0)	(5.5)†	(9.5)	(9.4)†	(9.5)
Day 90						
Control	61.4	13.8	24.7	—	38.5	—
(n = 8)	(11.8)	(2.4)	(9.9)		(11.8)	
Lomustine						
20 mg/kg	53.9	15.7	30.4	6.2	46.1	18.3
(n = 8)	(6.9)	(2.2)	(5.8)	(3.3)	(6.9)	(3.6)
50 mg/kg	23.6	34.4	40.6	17.5	76.4	41.1
(n = 7)	(9.2)†	(18.1)†	(14.3)*	(6.7)	(9.2)†	(7.4)

Animals were treated with a single dose of 20 or 50 mg/kg and sacrificed 30, 60 or 90 days following the administration. Cell cycle distributions were determined by flow cytometric analysis using propidium iodide.
Means (S.D.) of independent experiments.
*$P < 0.05$, †$P < 0.01$ vs. control (Kruskall-Wallis' test).

Table 5. Effect of carmustine on cell cycle progression capacity of rat hepatocytes

	% of cells in					
	G_0G_1	S	G_2 + M	G_2 + M(8c)	IP_1	IP_2
Day 30						
Control	66.6	18.1	15.3	—	33.5	—
(n = 8)	(4.1)	(5.6)	(3.9)		(4.3)	
Carmustine						
20 mg/kg	59.2	22.2	18.6	6.0	40.8	29.4
(n = 8)	(4.9)	(4.5)	(6.4)	(3.8)	(4.9)	(8.5)
50 mg/kg	41.6	19.3	29.2	10.4	59.6	22.1
(n = 8)	(8.7)†	(5.3)	(12.5)*	(5.5)	(8.4)†	(5.4)
Day 60						
Control	72.2	12.4	15.5	—	28.0	—
(n = 8)	(4.5)	(2.1)	(5.4)		(4.6)	
Carmustine						
20 mg/kg	43.6	17.1	39.3	17.0	56.4	29.8
(n = 4)	(5.6)†	(2.4)	(5.9)	(17.0)	(5.5)†	(11.4)
50 mg/kg	49.6	30.3	18.5	15.1	48.8	30.5
(n = 8)	(8.0)†	(14.3)*	(14.4)	(13.6)	(7.5)†	(7.3)
Day 90						
Control	61.1	14.6	24.3	—	38.9	—
(n = 8)	(5.8)	(3.2)	(4.5)		(5.8)	
Carmustine						
20 mg/kg	66.7	13.8	19.5	3.3	33.3	12.7
(n = 8)	(3.2)	(2.7)	(4.2)	(2.8)	(3.2)	(1.9)
50 mg/kg	63.6	12.1	23.5	7.1	37.6	19.1
(n = 8)	(7.5)	(3.1)	(5.9)	(5.9)	(6.7)	(4.4)

Means (S.D.) of independent experiments.
*$P < 0.05$, †$P < 0.01$ vs. control (Kruskall-Wallis' test).

observed, carmustine lesions may be partially reversible [9]; fotemustine induced discrete and reversible inflammatory lesions in portal tracts surrounding the bile ducts but no hepatotoxicity at the doses were used [9].

Brodie *et al.* [11] demonstrated *in vitro* that nitrosoureas inhibit the polymerisation of purified brain tubulin, which is the major microtubule protein of the mitotic apparatus [20]. Flow cytometric analysis confirmed that lomustine and carmustine induce accumulation of cells in G_2 + M phase, a moderate to high percentage of cells going to octaploidy, without significant decrease of S phase cells, or depletion of the quantity of cells in G_1 phase of cell cycle. These results are in accordance with those of several cultured cell lines exposed to nitrosoureas or to other various antineoplastic drugs, such as Chinese hamster cells in presence of bleomycine [21–23]. Tobey *et al.* [23] demonstrated that the major event was a mitotic non-disjunction, with an accumulation of cells in G_2 phase (4c content), and polyploidy due to a round of progression through the cell cycle without cell division. The latter event, among others, occurs with alkylating agents which are known to act on the mitotic spindle and to inhibit cytokinesis [24]. However, a similar effect was described with other types of antitumour drugs such as doxorubicin [25], cisplatin [26], bleomycin [27, 28] and cytarabine [29]. It was also demonstrated that cultured non-cycling cells were more sensitive to alkylating agents than are cycling cells. In general, the antitumour drugs had no effect on RNA or protein synthesis when cells have not entered into cycle.

Conversely, fotemustine-induced alterations of the cell cycle are not characterised by G_2 arrest, but by the accumulation of cells in S phase, which plateaued in the middle and late S phase. Rao [21] demonstrated that anticancer drug treatment has no effect on either RNA or protein synthesis as long as the cells have not completed a round of DNA synthesis after treatment. The cells that were in S phase at the time of treatment, or those that entered it subsequently, had difficulty in completing replication, as evidenced by an increase in the duration of S phase, as observed after fotemustine treatment.

This perturbation of DNA synthesis seems to be characteristic of alkylating agents [30–32]. Moreover, in our study, the coefficient of variation (CV) of the G_0G_1 population was greater in fotemustine-treated liver cells than in controls, CCNU or BCNU. This greater dispersion of cells in the G_0G_1 compartment could reflect heterogeneity of this cell population perhaps comprising cells arrested in early S phase: this could support the idea that fotemustine is a more potent and specific antineoplastic agent. The alkyating agents bind covalently to bases of DNA. The DNA crosslinking event is the main mechanism of antineoplastic properties of the nitrosoureas [23, 24], responsible for inhibition of DNA replication inducing an accumulation in S compartment particularly evidenced with fotemustine.

Table 6. Effect of fotemustine on cell cycle progression capacity of rat hepatocytes

	% of cells in					
	G_0G_1	S	G_2 + M	G_2 + M(8c)	IP_1	IP_2
Day 30						
Control	66.6	18.1	15.3	—	33.5	—
(n = 8)	(4.1)	(5.6)	(3.9)		(4.3)	
Fotemustine						
20 mg/kg	69.4	18.0	12.5	2.6	30.5	21.2
(n = 8)	(5.9)	(4.9)	(1.8)	(1.9)	(5.9)	(4.1)
50 mg/kg	65.0	30.2	7.4	—	33.2	—
(n = 6)	(10.0)	(15.2)	(4.4)		(10.4)	
Day 60						
Control	72.2	12.4	15.5	—	28.0	—
(n = 8)	(4.5)	(2.1)	(5.4)		(4.6)	
Fotemustine						
20 mg/kg	63.7	16.4	19.8	10.1	36.3	26.8
(n = 8)	(7.2)	(4.8)	(6.0)	(2.8)	(7.2)	(3.5)
50 mg/kg	53.9	30.7	15.4	8.5	44.7	29.0
(n = 8)	(9.7)*	(9.7)†	(4.2)	(6.2)	(9.7)*	(9.6)
Day 90						
Control	61.1	14.6	24.3	—	38.9	—
(n = 8)	(5.8)	(3.2)	(4.5)		(5.8)	
Fotemustine						
20 mg/kg	65.9	15.0	19.1	14.3	34.1	27.0
(n = 8)	(3.5)	(4.0)	(4.4)	(6.5)	(3.5)	(7.0)
50 mg/kg	59.3	21.2	18.8	13.2	40.7	24.7
(n = 4)	(5.8)	(3.8)	(3.4)	(5.0)	(7.0)	(2.2)

Means (S.D.) of independent experiments.
*P < 0.05, †P < 0.01 vs. control (Kruskall-Wallis' test).

Chloroethylnitrosoureas are reactive compounds whose metabolism undergoes chemical degradation with (i) two minor pathways, including denitrosation and oxidative dechlorination (formation of inactivated metabolites), (ii) spontaneous generation of alkylating intermediate products (chloroethyl carbonium ions), (iii) carbamlyation which yields organic isocyanates.

These metabolic pathways are similar for the nitrosoureas lomustine, carmustine and fotemustine, but their respective roles differ in regard to occurrence of metabolites. The role of the different pathways involved in fotemustine metabolism is under investigation. Nevertheless, the two major metabolites identified in urinary profiles of man, rat and mouse come from the oxidative dechlorination pathway and have no cytotoxic activity [33].

Even though lomustine and carmustine are classified as alkylating agents, their cytotoxicity is associated of an inhibitory effect on the mitotic spindle. Indeed, if the antitumour activity of nitrosoureas is mainly imputed to the alkylation of DNA, carbamylation of intracellular protein functional groups by isocyanates has been hypothesised to contribute to side effects and toxicity [34–36].

Lomustine, carmustine and their respective derived isocyanates, 2-chloroethylisocyanate (CEI) and cyclohexylisocyanate (CHI), with the 4-OH metabolites of lomustine exhibited an inhibitory effect on protein synthesis: a good correlation has been found between esterase inhibition in intact murine cells and the chemical carbamylating activity [37, 38]. The compounds which were unable to form isocyanates were mainly inactive.

In vivo, the occurrence of these metabolites varies and may explain the discrepancy in toxicity between lomustine and carmustine. Our study shows a correlation between the intensity of hepatotoxic effects of nitrosoureas and respective cycle alterations in regard of the role played by isocyanates metabolites. The more severe hepatotoxic effects, in particular cholestatic, are noted with lomustine and carmustine: studies described a similar canalicular target for the two nitrosoureas [10, 39], early event followed by a decrease in microsomal mixed function oxygenase and cytochrome P-450 activities [40]. In contrast, fotemustine is devoid of hepatotoxicity and is without evidence of such toxic metabolites.

The liver cell cycle alterations induced by lomustine and carmustine are the more severe and predominantly reflected by inhibition of G_2 phase. A similar alteration was noted in an *in vitro* EMT6 cell model with lomustine [41]. On the contrary, in presence of fotemustine, the lower impact on G_2 cells through the absence of effect on microtubule system, is corroborated with the morphological and biochemical observations: fotemustine lacks of morphological alterations of biliary ducts and of ultrastructural alterations. Since the microtubule protein, tubulin, plays a key role in dividing cells, we suggest that the cell cycle alterations expressed by a G_2 phase arrest might be primarily related to isocyanates.

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Prolonged Bleeding Time Due to Mitotane Therapy

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After finding prolonged bleeding times in 2 patients treated with mitotane, we prospectively studied 7 patients with adrenocortical cancer on mitotane therapy. Before and 1 and 2 or more weeks after starting mitotane we determined the platelet counts, bleeding times and global coagulation parameters. All patients had a normal bleeding time before treatment. In 6 cases the bleeding time became prolonged (245-555 s). 4 patients exhibited platelet aggregation responses compatible with an aspirin-like defect. It is concluded that mitotane may cause a clinically relevant defect of platelet function.
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INTRODUCTION

ADRENAL CORTICAL carcinoma is an uncommon tumour in man. Mitotane is the drug of choice for patients with inoperable, recurrent and metastatic disease [1]. In our hospital mitotane is given even after apparently radical surgery because of frequent recurrences and the assumption that cytotoxic therapy is more effective when the tumour load is low [2]. Mitotane therapy is continued for 2 years if resection has been judged to be complete or for 1 year after apparent disappearance of the tumour. Serum trough concentrations of mitotane exceeding 14 mg/l were associated with a response rate of 50%, whereas no therapeutic effect was seen in patients with levels lower than 10 mg/l [2]. We therefore aim to achieve serum trough levels above 14 mg/l (when possible 25 mg/l). The mechanism of action of mitotane