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Use of Protease Inhibitors to Improve Calcitonin Absorption from the Small and Large Intestine in Rats

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Abstract

The objective of this study was to examine the effects of protease inhibitors on the absorption of calcitonin from different regions of the intestine in rats. The absorption experiments were investigated by in-situ use of closed intestinal loops in rats and stability of calcitonin was examined in mucosal homogenates and intestinal fluids. The intestinal absorption of calcitonin was evaluated by measurement of its hypocalcaemic effect.

No substantial hypocalcaemic response was observed when calcitonin was administered into the jejunum or colon. A slight hypocalcaemic effect was observed after administration of calcitonin into the ileum. Of the co-administered protease inhibitors, bacitracin (20 mM) strongly promoted calcitonin absorption from the jejunum, ileum and colon. A significant hypocalcaemic effect was also obtained after intestinal administration of calcitonin with soybean trypsin inhibitor (10 mg mL^{-1}), camostat mesylate (20 mM) or aprotinin (2 mg mL^{-1}). In the stability experiment, bacitracin reduced the degradation of calcitonin in the different intestinal homogenates. Soybean trypsin inhibitor significantly reduced the degradation of calcitonin in the fluids of the small intestine. We also examined the different endopeptidases in gut luminal fluids and the different exopeptidases in gut mucosal homogenates of rats. The ranking order for the total endopeptidase activity of the intestinal fluids was jejunum > ileum > colon. That for total exopeptidase activity of the intestinal mucosa was jejunum > ileum > colon.

These results suggest that endo- and exopeptidases might be responsible for the hydrolysis of calcitonin and that protease inhibitors might usefully improve absorption of calcitonin to the systemic circulation from the large intestine.

Peptides and proteins such as insulin and calcitonin are usually given only by injection, because when taken orally they are degraded by the proteolytic enzymes in the gastrointestinal tract or cannot permeate the intestinal mucosa because of their hydrophilicity and large molecular size. Different approaches, such as alternative routes (Morimoto et al 1991; Yamamoto et al 1994b), absorption enhancers (Hirai et al 1981), protease inhibitors (Okagawa et al 1994; Yamamoto et al 1994a), chemical modification (Yodoya et al 1994; Asada et al 1995) and dosage forms, have been examined to overcome the problems of delivering these peptides and proteins via the gastrointestinal tract. Of these approaches, the use of protease inhibitors has been shown to improve the small and large intestinal absorption of peptides. Morishita et al (1993) reported that the effect of aprotinin on the intestinal absorption of insulin was site-dependent. Yamamoto et al (1994a) reported that different protease inhibitors including bacitracin, camostat, and sodium glycocholate effectively improved insulin absorption from the gastrointestinal tract.

In man calcitonin is a 32-amino-acid hormone of molecular weight approximately 3400 Da produced in C-cells of the thyroid gland. The physiological activity of calcitonins is to reduce plasma Ca^{2+} concentrations and they are used in the treatment of disease such as osteoporosis and Paget's disease (Greenberg et al 1974). Calcitonin is usually given by injection only (as is insulin) because when taken orally it is degraded by the proteolytic enzymes in the gastrointestinal tract or cannot penetrate the intestinal mucosa because of its hydrophilicity and large molecular size (Nishihata et al 1986).

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In this study, therefore, to improve the intestinal absorption of calcitonin we investigated the effects of different protease inhibitors on the absorption of calcitonin from the rat small and large intestines. We also examined the effects of these protease inhibitors on the stability of calcitonin in homogenates of small and large intestinal mucosae and intestinal fluid. Finally, the activities of proteases, which might be responsible for calcitonin hydrolysis, were determined in the intestinal mucosae and intestinal fluids.

Materials and Methods

Chemicals

Calcitonin from man was a gift from Suntory (Osaka, Japan). Aprotinin, 7-amino-4-methyl coumarin, succinyl (Suc)-Ala-Pro-Ala-4-methylcoumaryl-7-amide (MCA) and *t*-butyloxycarbonyl (Boc)-Gln-Ala-Arg-MCA were purchased from Sigma (St Louis, MO). Bacitracin, soybean trypsin inhibitor and the calcium C test Wako were obtained from Wako Pure Chemical (Osaka, Japan). Arg-MCA, Leu-MCA and Ala-MCA were obtained from the Peptide Institute (Osaka, Japan). Camostat mesylate was a gift from Ono Pharmaceutical (Osaka, Japan). All other chemicals and solvents were of reagent grade.

In-situ closed-loop methods

Absorption experiments were performed by the in-situ closed-loop method (Hashizume et al 1991; Yamada et al 1992). Male Wistar albino rats, 240–300g, were anaesthetized with intraperitoneal sodium pentobarbital ($32\,mg\,kg^{-1}$). Animals were fasted for approximately 16h before the experiments but water was freely available. The intestine was exposed through a midline abdominal incision and 10-cm jejunum, ileum or colon loops were prepared by cannulation with silicone tubing (i.d. 3mm; o.d. 5mm) at the proximal and distal ends of the loops. The luminal surface of the loop was washed with isotonic phosphate buffer (20mL). Calcitonin was dissolved in isotonic phosphate buffer at pH 7·4 to a final concentration of $100\,\mu g/2\,mL\,kg^{-1}$. Protease inhibitors such as aprotinin ($2\,mg\,mL^{-1}$), bacitracin (20mM), soybean trypsin inhibitor ($10\,mg\,mL^{-1}$) or camostat mesylate (20mM) were added to the dosing solutions. The doses of the different protease inhibitors used in these experiments were decided from the doses used in experiments on the absorption of insulin via the gastrointestinal mucosae in our previous study (Yamamoto et al 1994a). The drug solution was warmed to 37°C and 2mL was injected into the jejunum, ileum or colon loop. Calcitonin solution from man was administered intravenously by bolus injection (0·1 μg/rat) for calculation of the pharmacological availability (%) in each experiment.

Preparation of homogenates of the intestinal mucosae

Mucosal tissue homogenates were prepared as previously described (Yamamoto et al 1990). Briefly, twelve Wistar albino rats, 240–300g, were anaesthetized with sodium pentobarbital ($32\,mg\,kg^{-1}$, i.p.). The animals were fasted for approximately 16h before the experiments, but water was freely available. After washing of the luminal surface with saline solution the jejunal, ileal and colonic mucosae were removed by scraping the epithelial cell layers. These specimens were pooled by tissue type and stored at −80°C. Immediately before each experiment, specimens were thawed at room temperature and then homogenized in isotonic phosphate buffer (pH 7·4; 1–2mL) at 4°C by use of a Polytron homogenizer (Kinematica, Switzerland). The homogenate was centrifuged at 5000*g* in a refrigerated (4°C) centrifuge for 10min to remove cellular and nuclear debris. The resulting supernatant was diluted with isotonic phosphate buffer to a protein concentration of $10\,mg\,mL^{-1}$, as determined by the method of Lowry et al (1951) with bovine serum albumin as the standard.

Preparation of the intestinal fluids

Fluids from the jejunum, ileum or colon were collected from the rats according to the method of Takada & Ushirogawa (1991). Male Wistar albino rats, 240–300g, fasted for approximately 16h before the experiments but with water freely available, were anaesthetized with sodium pentobarbital ($32\,mg\,kg^{-1}$, i.p.). After injection of phosphate-buffered saline (PBS; 2·5mL) into the jejunum, ileum or colon, they were cannulated with silicone tubing (3mm i.d. × 5mm o.d.) at the proximal and distal ends of the intestinal loop. The rats were left for 5min and their intestinal contents were washed out by infusing 5mL of PBS and the eluate was collected. This eluate was extracted with 5 × 2 vols dichloromethane to remove any lipids that might have interfered with the analysis of calcitonin by high-performance liquid chromatography (HPLC). This extract was diluted with PBS to yield a protein concentration of $10\,mg\,mL^{-1}$ and was subsequently used as the intestinal fluid (Okagawa et al 1994).

In-vitro stability experiments

The degradation of calcitonin was studied by incubating the luminal fluids or homogenate of the

intestinal mucosae (240 μL), which had been pre-incubated at 37°C for 15 min, with calcitonin solution (0·02 mM; 360 μL) in the presence or absence of a given concentration of aprotinin (1 mg mL^{-1}), bacitracin (20 mM), soybean trypsin inhibitor (10 mg mL^{-1}), or camostat mesylate (20 mM). At predetermined times until 15 min, 50-μL samples were withdrawn from the incubation mixture and acetic acid (50%; 100 μL) was added to terminate the reaction. The resulting mixture was centrifuged at 10000 rev min^{-1} for 5 min to remove the precipitated proteins. These samples were analysed by HPLC (Yamamoto et al 1994a).

Determination of the enzyme activity in the intestinal mucosae and intestinal fluids

Enzyme activity was assayed as described by Ekstrom & Westrom (1991). Assay of the activity of the different proteases is based on fluorimetric measurement of 7-amino-4-methylcoumarin liberated from the fluorogenic substrate, peptidyl-MCA. The incubation mixture consisted of phosphate-buffered saline (pH 7·4), a substrate, and the homogenate of the intestinal mucosae or the intestinal fluid. The activity of aminopeptidase B, leucine aminopeptidase, aminopeptidase N, elastase, chymotrypsin and trypsin were examined with Arg-MCA as substrate and KCl as activator, with Leu-MCA or Ala-MCA plus $CaCl_2$, with Suc-Ala-Pro-Ala-MCA plus $CaCl_2$, with Suc-Ala-Ala-Pro-Phe-MCA plus $CaCl_2$, and with *t*-Boc-Gln-Ala-Arg-MCA. Trichloroacetic acid (50%) was added to terminate the reaction and the resulting mixture was centrifuged for 5 min to remove precipitated protein. The 7-amino-4-methylcoumarin released into the supernatant was determined by spectrofluorimetry (Hitachi, F-2000); the excitation and emission wavelengths were 380 nm and 440 nm, respectively. Protease activity (units) was calculated from specific protease enzyme activity (μmol min^{-1} (mg protein)$^{-1}$) × protein concentration in the homogenate of the intestinal mucosae or intestinal fluid (mg mL^{-1}) × volume of the homogenate from the intestinal mucosae or intestinal fluid (mL).

Analytical methods

The intestinal absorption of calcitonin was estimated by measurement of its hypocalcaemic effect. For determination of the Ca^{2+} concentration in plasma, blood samples (200 μL) were taken periodically from the left jugular vein after dosing, centrifuged at 5000 rev min^{-1} for 5 min, and the plasma samples were collected. The plasma Ca^{2+} concentrations were determined by means of the Wako Calcium C test. The decrease in plasma calcium level (A%) was calculated by a modification of the method of Hirai et al (1981) using the equation:

$$A\% = [1 - AUC_{0\rightarrow240}/(100\% \times 240\ \text{min})] \times 100$$

where the area above the 100% line was ruled out for calculating $AUC_{0\rightarrow240}$, the area under the plasma concentration–time curve between 0 and 240 min. 100% was defined as the initial Ca^{2+} concentration for each rat. The A% value was 8·14% when the calcitonin solution (0·1 μg/rat) was intravenously administered by bolus injection.

The pharmacological availability (%) was calculated by use of the equation:

$$\text{Pharmacological availability (\%)} = (\text{gastrointestinal A\%}/\text{intravenous A\%}) \times (\text{intravenous dose}/\text{gastrointestinal dose}) \times 100$$

The stability of calcitonin was assayed by reversed-phase HPLC on a Vydac protein and peptide C_{18} column (150 mm × 4·6 mm, 5 μm). HPLC was performed with a Waters LC Module 1 and a Shimadzu model C-R4A integrator. The mobile phase was a mixture of acetonitrile and water containing 0·1% trifluoroacetic acid adjusted to pH 3 with phosphoric acid; the flow rate was 1·1 mL min^{-1}. The proportion of acetonitrile in the mobile phase was increased linearly from 10 to 34% during the first 23 min, maintained at 34% for the next 11 min, and then increased linearly to 40% during another 6 min. The retention time of calcitonin was approximately 23 min. Calcitonin eluting from the column was detected by UV absorption at 210 nm.

Statistical analysis

Results are expressed as means ± s.e.; statistical analysis was performed by use of Student's *t*-test.

Results

Effects of different protease inhibitors on the intestinal absorption of calcitonin

No significant changes in plasma calcium concentration were observed when isotonic phosphate buffer was administered to the jejunum or colon (data not shown). Figure 1 shows the time-course of the calcium concentration in the plasma after intestinal administration of calcitonin from man (100 μg kg^{-1}), in the absence or presence of different protease inhibitors, into different intestinal segments. Table 1 summarizes the reduction in plasma calcium levels (A%) and the pharmacological availability (%) of calcitonin after its administration with these protease inhibitors into

the jejunum, ileum or colon. When calcitonin alone was administered into the jejunum or colon, no substantial hypocalcaemic response was noted during 4h of observation, although we observed a slight hypocalcaemic effect after administration of calcitonin into the ileum. However, in the presence of different protease inhibitors significant hypocalcaemic effects were observed after administration of calcitonin into the jejunum, ileum and colon. The hypocalcaemic effects in the presence of these protease inhibitors were more apparent in the colon than in the jejunum or ileum. Of the protease inhibitors investigated bacitracin most effectively improved the stability of calcitonin in all regions of the intestine. The largest hypocalcaemic effect was observed after administration of calcitonin with bacitracin into the colon.

Stability of calcitonin in homogenates of the intestinal mucosae

The effects of different protease inhibitors on the degradation of calcitonin in homogenates of the jejunal, ileal or colonic mucosae are shown in Figure 2. The disappearance of calcitonin followed first-order kinetics. Calcitonin was rapidly degraded in homogenates of these intestinal mucosae, especially in the jejunal and ileal mucosal homogenates without any protease inhibitor. However, aprotinin, soybean trypsin inhibitor, camostat and bacitracin effectively reduced the degradation of

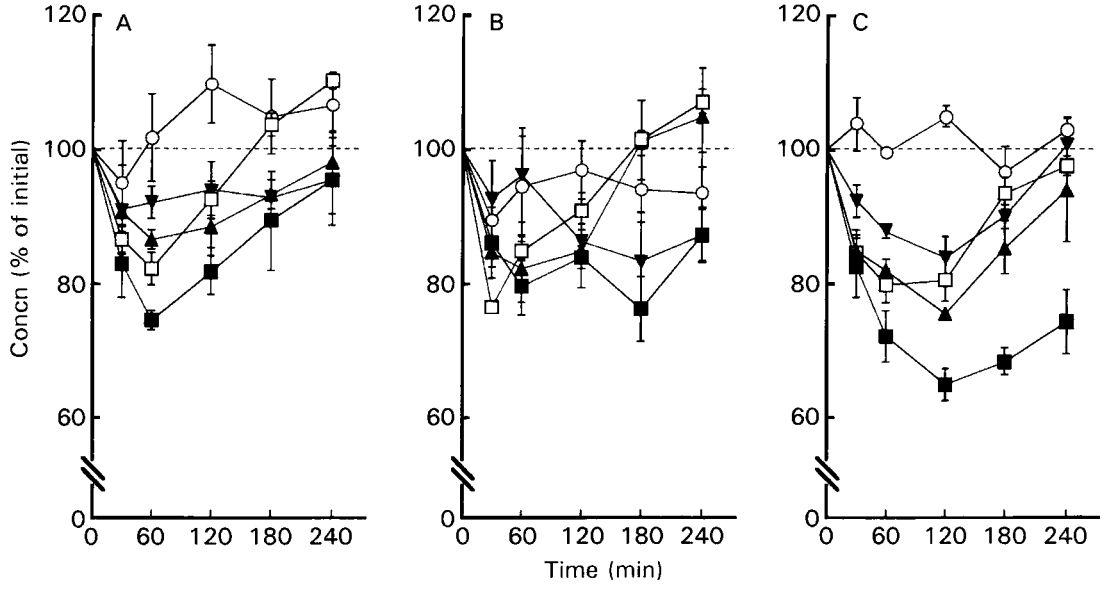

Figure 1. Concentration–time profiles of calcium in plasma after administration of calcitonin from man and of protease inhibitors (○, control; □, aprotinin 2mg mL^{-1}; ▼, soybean trypsin inhibitor 10mg mL^{-1}; ▲, camostat 20mM; ■, bacitracin 20mM) into different intestinal segments (A, jejunum; B, ileum; C, colon). Data are expressed as means ± s.e. of results from four experiments.

Table 1. Effects of protease inhibitors on the absorption of calcitonin from man in different regions of the rat intestine.

	Jejunum		Ileum		Colon	
	Decrease in plasma calcium (%)	Pharmacological availability (%)	Decrease in plasma calcium (%)	Pharmacological availability (%)	Decrease in plasma calcium (%)	Pharmacological availability (%)
Control	2·44 ± 1·11	0·15	6·35 ± 1·92	0·40	1·40 ± 0·69	0·08
Aprotinin (2mg mL^{-1})	7·01 ± 1·14*	0·44	7·53 ± 0·98	0·47	12·72 ± 0·88†	0·79
Soybean trypsin inhibitor (10mg mL^{-1})	7·14 ± 2·64*	0·45	13·01 ± 3·54*	0·81	11·43 ± 0·84†	0·71
Camostat (20mM)	8·80 ± 1·43*	0·55	11·99 ± 3·26*	0·75	17·80 ± 1·75†	1·11
Bacitracin (20mM)	15·86 ± 1·44†	0·99	17·49 ± 2·86*	1·09	27·40 ± 0·63‡	1·71

Values for the decrease in plasma calcium are means ± s.e. of results from four rats. *$P < 0·05$, †$P < 0·01$, ‡$P < 0·001$, significantly different from control result.

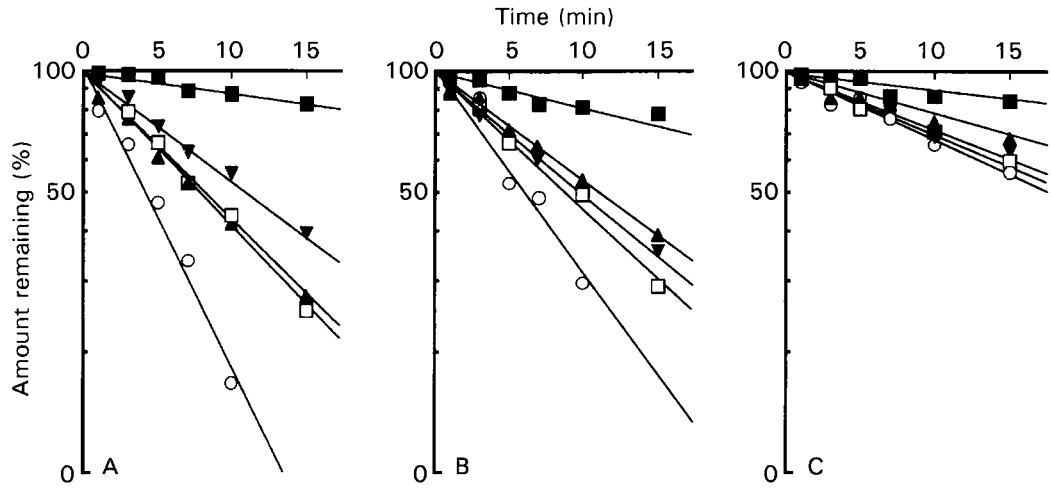

Figure 2. Effects of different protease inhibitors (○, control; □, aprotinin 1 mg mL^{-1}; ▼, soybean trypsin inhibitor 10 mg mL^{-1}; ▲ camostat 20 mM; ■, bacitracin 20 mM) on the degradation of calcitonin from man by homogenates of different intestinal mucosae (A, jejunum; B, ileum; C, colon). Data are expressed as means ± s.e. of results from three experiments.

Table 2. Effects of protease inhibitors on the half-lives of hydrolysis of calcitonin from man in homogenates of the different intestinal mucosae.

	Jejunum		Ileum		Colon	
	Half-life (min)	Ratio	Half-life (min)	Ratio	Half-life (min)	Ratio
Control	4·08 ± 0·93	1·00	5·59 ± 0·92	1·00	19·23 ± 2·14	1·00
Aprotinin (1 mg mL^{-1})	7·87 ± 1·45	1·93	8·42 ± 1·35	1·51	24·77 ± 1·12	1·29
Soybean trypsin inhibitor (10 mg mL^{-1})	11·14 ± 1·14*	2·73	10·29 ± 1·88	1·84	21·39 ± 3·21	1·11
Camostat (20 mM)	8·47 ± 1·01*	2·08	10·59 ± 2·11	1·89	39·96 ± 2·97*	2·08
Bacitracin (20 mM)	50·75 ± 3·21‡	12·4	40·99 ± 5·12†	7·33	72·49 ± 8·21†	3·77

Half lives are means ± s.e. of results from three experiments. *$P < 0·05$, †$P < 0·01$, ‡$P < 0·001$, significantly different from control result.

calcitonin in all the homogenates, bacitracin being the most effective in all the intestinal regions. Table 2 summarizes the effects of protease inhibitors on the half-life of calcitonin hydrolysis in intestinal mucosal homogenates of the jejunum, ileum and colon. Bacitracin substantially reduced the degradation of calcitonin in the jejunal, ileal and colonic intestinal mucosae. Aprotinin, soybean trypsin inhibitor and camostat moderately inhibited the degradation of calcitonin in these regions. The maximum reduction in proteolytic cleavage of calcitonin was seen in the presence of bacitracin in the colonic mucosae.

Stability of calcitonin in small intestinal fluids

When the luminal surface was not washed with saline solution no hypocalcaemic effect was observed after administration of calcitonin into each region, even in the presence of protease inhibitors, implying that calcitonin was degraded by luminal enzymes even in the presence of protease inhibitors under unwashed conditions.

To confirm the contribution of luminal enzymes to calcitonin proteolysis we examined the effects of protease inhibitors on the stability of calcitonin in the fluids of the small intestine. Table 3 shows the degradation half-life for calcitonin proteolysis in the fluids of the small intestine in the presence or absence of protease inhibitors. Calcitonin was rapidly degraded in the fluids of the small intestine, suggesting that it might be metabolized by proteolytic enzymes such as elastase, chymotrypsin, and trypsin. Soybean trypsin inhibitor significantly reduced the degradation of calcitonin in the fluids.

Activity of proteases in the intestinal fluids and intestinal mucosae of the rat

Figure 3 shows the activity of proteases in the intestinal fluids and intestinal mucosae of the rat. Because our previous study revealed no aminopeptidases in the intestinal fluids and little endopeptidase activity in the intestinal mucosae, in this study we examined the major endopeptidases

Table 3. Effect of protease inhibitors on the half-lives of hydrolysis of calcitonin from man in the small intestinal fluids of rats.

	Half-life (min)	Ratio
Control	0·41 ± 0·02	1·00
Camostat (20 mM)	2·01 ± 0·44*	4·90
Soybean trypsin inhibitor (10 mg mL^{-1})	24·0 ± 2·31†	58·5

Half lives are means ± s.e. of results from three experiments. *$P < 0·05$, †$P < 0·01$, significantly different from control result.

including elastase, chymotrypsin and trypsin in intestinal fluids and the major exopeptidases, including aminopeptidase B, leucine aminopeptidase and aminopeptidase N, in the intestinal mucosae. The ranking order of the total endopeptidase activity of the intestinal fluids was jejunum > ileum > colon. For the total exopeptidase activity of the intestinal mucosae the ranking order was jejunum > ileum > colon.

Discussion

No substantial hypocalcaemic response was observed when calcitonin alone was administered into the jejunum or colon, a result consistent with the report by Hastewell et al (1994) that the bioavailability of intracolonically administered calcitonin from man was less than 1%. Presumably, this result is attributable to the extensive metabolism of calcitonin in the intestinal fluids and mucosae, as was apparent from Figure 2 and Tables 2 and 3. Another possible reason for the low bioavailability of calcitonin is its poor membrane permeability because of its large molecular size and low lipophilicity.

A slight hypocalcaemic effect was found after administration of calcitonin into the ileum. Morishita et al (1993) reported a clear decrease in glucose level in the ileum only when insulin alone was administered to different intestinal loops. It has recently been proposed that M cells in the Peyer's patches are the major site of pinocytosis of macromolecules including insulin and calcitonin (Kararli 1989) and it is, therefore, possible that the morphological features of Peyer's patches lead to much greater absorption of calcitonin from the ileum than from the other regions of the gastrointestinal tract, although we have not examined the transport of calcitonin across the M cells in the Peyer's patches.

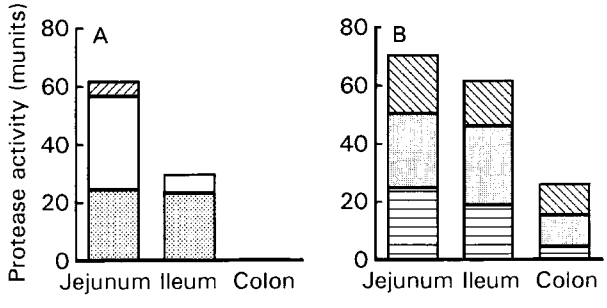

Figure 3. Net activities of different endopeptidases in gut luminal fluids (A) and of different exopeptidases in gut mucosal homogenates (B) of rats: ▨, elastase; □, chymotrypsin; ▦, trypsin; ▧, aminopeptidase B; ▢, leucine aminopeptidase; ▤, aminopeptidase N. Data are expressed as means ± s.e. of results from three experiments.

In this study we observed that the overall effects of protease inhibitors were more apparent in the large intestine than in the small intestine. This result concurs with our previous findings, which showed that absorption of insulin from the large intestine was much greater than from the small intestine in the presence of protease inhibitors (Yamamoto et al 1994a; Tozaki et al 1996). This might be related to the different activity of enzymes responsible for insulin hydrolysis in the small and large intestines. Indeed, our current study has indicated that the activity of exo- and endopeptidases in the small intestinal fluid and mucosa was much higher than in the large intestine. Our pilot study also revealed no significant hypoglycaemic response, even in the presence of protease inhibitors, unless the luminal surface of the small intestine had been washed. Therefore, we consider that luminal enzymes also play an important role in the degradation of insulin in intestinal fluids. This speculation was supported by the rapid degradation of insulin in the luminal fluid in the absence of protease inhibitor.

Bacitracin was the most effective protease inhibitor of those studied in this experiment. Bacitracin, a cyclic polypeptide antibiotic obtained from *Bacillus licheniformis*, has a variety of biological activity, including inhibition of peptidoglycan synthesis, mammalian transglutaminase activity, and several proteolytic enzymes including subtilisin, papain and leucine aminopeptidase (Roth 1981). Because of this inhibitory activity, bacitracin has been used to inhibit the degradation of peptides and proteins such as insulin, buserelin and luteinizing hormone (Raehs et al 1988; Yamamoto et al 1994b). This study has also demonstrated that the degradation of calcitonin was significantly inhibited by the addition of bacitracin to homogenates of jejunal, ileal and colonic mucosae, and so the positive result obtained for bacitracin in the absorption studies might be attributable to its inhibition of proteolytic enzymes in the intestine. Our previous study indicated that intestinal

absorption of phenol red and fluorescein isothiocyanate-labelled dextran with an average molecular weight of 4000, stable and water-soluble compounds, was improved by addition of bacitracin (Gotoh et al 1996). It might thus be possible that the intestinal absorption of calcitonin was improved by bacitracin because of its absorption-enhancing action in addition to its inhibitory effect on proteases.

Our current study also indicated that aprotinin, soybean trypsin inhibitor and camostat improved the intestinal absorption of calcitonin, although their enhancing effects were less than that of bacitracin. It has been reported that the activities of trypsin and chymotrypsin were reduced by addition of aprotinin and soybean trypsin inhibitor (Saffran et al 1988) and the degradation of calcitonin was reduced by addition of these protease inhibitors in the current study. Consequently, the proteolytic inhibitory effects of these protease inhibitors might be related to their enhancement of the intestinal absorption of calcitonin. It is known that camostat mesylate inhibits the activity of aminopeptidases and proteases such as trypsin, plasmin and kallikrein (Morimoto et al 1991) and the improved stability and absorption of calcitonin by camostat might, therefore, be attributable to this inhibitory action.

In this study we found that soybean trypsin inhibitor effectively reduced the degradation of calcitonin in small intestinal fluids and that the activity of endopeptidases such as trypsin and chymotrypsin were relatively high in small intestinal fluid, although we found almost no aminopeptidase activity in the fluids. Consequently, it might be considered that soybean trypsin inhibitor inhibits the activity of endopeptidases such as trypsin and chymotrypsin responsible for the hydrolysis of calcitonin, thereby reducing the degradation of calcitonin in the small intestinal fluids.

In studies of enzymatic activity in the intestinal mucosae and fluids, we found that the activity of endopeptidases and exopeptidases was higher in the jejunum and ileum than in the colon. Bai (1993) reported that the activity of angiotensin converting enzyme and endopeptidase–24·11 was higher in the small intestine than in the large intestine and Hayakawa & Lee (1992) reported that the activity of several proteases was usually higher in the small intestine than in the large intestine. Our present findings are, therefore, in agreement with the previous results and suggest that the large intestine could be a useful site for administration of larger molecular peptides including calcitonin and insulin.

Although we have not determined which proteases were mainly responsible for the hydrolysis of calcitonin observed in this study, Yokoo et al (1988) reported that high molecular-weight peptides such as calcitonin and insulin were first metabolized to small peptides by endopeptidases in the small intestinal fluids and then further metabolized by aminopeptidases in the intestinal mucosae. We therefore consider that both endo- and exopeptidases might be responsible for the hydrolysis of calcitonin in the intestine.

In conclusion, we have demonstrated that the effects of protease inhibitors on the intestinal absorption of calcitonin were site-dependent. Protease inhibitors such as bacitracin and camostat effectively improved the absorption of calcitonin from the intestine, especially the large intestine. Furthermore, the degradation of calcitonin was inhibited by these protease inhibitors in the intestinal homogenates and fluids; this might be related to their absorption-enhancing effect on calcitonin. These results suggest that these protease inhibitors might be useful for improving the large intestinal absorption of calcitonin to the systemic circulation.

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Influence of Trimebutine on Inflammation- and Stress-induced Hyperalgesia to Rectal Distension in Rats

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Abstract

The effects of trimebutine and its major metabolite, *N*-desmethyltrimebutine on inflammation- and stress-induced rectal hyperalgesia have been evaluated in rats fitted with electrodes implanted in the longitudinal striated muscle of the abdomen.

Intermittent rectal distension was performed before and 3 days after induction of rectal inflammation by local infusion of trinitrobenzenesulphonic acid (in ethanol). Stress consisted of 2h partial restraint and rectal distension was performed before and 30min after the end of the partial restraint session. The animals were treated intraperitoneally with trimebutine or desmethyltrimebutine (5, 10 or 20mgkg^{-1}) or vehicle 15min before rectal distension. Naloxone (1mgkg^{-1}) or saline was injected subcutaneously before trimebutine and desmethyltrimebutine. Before treatment trimebutine at the highest dose (20mgkg^{-1}) reduced the abdominal response to rectal distension for the highest volume of distension (1·6mL) whereas desmethyltrimebutine was inactive. After rectocolitis the abdominal response to rectal distension was enhanced and trimebutine at 5mgkg^{-1} reduced and at 10mgkg^{-1} suppressed inflammation-induced hyperalgesia, an effect reversed by naloxone. Desmethyltrimebutine was inactive. Stress-induced hypersensitivity was attenuated or suppressed, or both, by trimebutine and desmethyltrimebutine at doses of 5, 10 or 20mgkg^{-1}; greater efficacy was observed for desmethyltrimebutine and the effects were not reversed by naloxone.

It was concluded that trimebutine and desmethyltrimebutine are active against inflammation- and stress-induced rectal hyperalgesia but act differently. The effect of trimebutine on inflammation-induced hyperalgesia is mediated through opioid receptors.

Abdominal pain is the major symptom of functional bowel disorders, and reduced threshold to rectal sensation and pain is commonly observed in patients with irritable bowel syndrome (Chaudhary & Truelove 1962; Ritchie 1973; Lembo et al 1994). Even though the existence of gut inflammatory reactions in irritable bowel syndrome is still a subject of controversy, recent reports have emphasized the possibility that previous experience of gut inflammation such as gastroenteritis might trigger the delayed occurrence of irritable bowel syndrome particularly in patients subsequently subject to a stressful life (Gwee et al 1996).

Inflammatory bowel disease is associated with reduced threshold of pain sensation to colonic or rectal distension in man (Rao et al 1987). Colonic or rectal irritation with chemicals induces a drastic increase in barosensitivity to gradual rectal distension in rats (Ness et al 1991; Morteau et al 1994a). Acute physical and mental stress stimuli are known to affect the perception of pain related to barosensitivity in both healthy subjects and irritable bowel syndrome patients (Erckenbrecht et al 1988; Ford et al 1995; Métivier et al 1996). Recently, it has been demonstrated that restraint stress acts in the opposite direction to somatic and visceral sensitivity with a transient lowering of the threshold of rectal distension-induced abdominal cramps in rats (Porro & Carli 1988; Gué et al 1997).

Opioid substances with activity at μ (morphine, Dago) or κ (fedotozine, U50488) receptors have previously been shown to be active on pseudo-affective (cardiovascular) response to colonic distension (Diop et al 1994; Danzebrink et al 1995), with a greater efficacy in the presence of acetic acid-induced colitis (Langlois et al 1994). It has

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also been reported that mixed μ and κ opioid substances, for example trimebutine, might prevent stress-induced alterations of upper (Gué et al 1988) and lower (Junien et al 1991) gut motility in dogs and rats, respectively.

This aim of this work was to determine whether trimebutine, a classical drug used for the treatment of irritable bowel syndrome and which has mixed μ and κ agonist activity (Valori & Shannon 1987; Pascaud et al 1989) has different effects on rectal sensitivity to distension before and after inflammation or stress. We have also investigated whether such activity is linked to activation of opioid receptors, both by use of naloxone and by comparing its effects with those of its major metabolite *N*-desmethyltrimebutine, a weak opioid agonist.

Material and Methods

Chemicals

Trimebutine maleate and *N*-desmethyltrimebutine were provided by the Institut de Recherches Jouveinal (Fresnes, France) and naloxone hydrochloride was purchased from Sigma (La Verpillère, France).

Animal preparation

Sixteen groups of eight male Wistar rats (Elevage Janvier, Le Genest Saint Isle, France), 250–350 g, were surgically prepared for electromyography, according to a previously described technique (Rukebusch & Fioramonti 1975). Rats were anaesthetized with acepromazine (0·3 mL, 0·5 mg kg^{-1}) and ketamine (Imalgene 1000, Rhône-Mérieux, Lyon, France; 0·3 mL, 120 mg kg^{-1}) injected intraperitoneally. One group of three electrodes was implanted in the abdominal external oblique musculature just above the inguinal ligament. Electrodes were exteriorized on the back of the neck and protected by a glass tube attached to the skin.

Motility recordings

Electromyographic recording began five days after surgery. The electrical activity of the abdominal striated muscles was recorded with an electroencephalograph (Mini VIII, Alvar, Paris, France) using a short time constant (0·03 s) to remove low-frequency signals (< 3 Hz).

Induction of colitis

Trinitrobenzenesulphonic acid (80 mg kg^{-1} in 0·3 mL 50% ethanol) was administered intrarectally through a silicone rubber catheter introduced 1 cm into the anus under light ether anaesthesia, as described previously (Morteau et al 1994a).

Stress procedure

Partial-restraint stress, a relatively mild, non-ulcerogenic model of restraint (Williams et al 1988), was used. Briefly, the animals were lightly anaesthetized with diethyl ether and their fore-shoulders, upper forelegs and thoracic trunk were wrapped in a confining harness of paper tape to restrict, but not to prevent body movement. The animals were then placed in their home cage for 2 h. The rats recovered from the diethyl ether within 2–3 min and immediately moved about in their cages and ate and drank, but the mobility of their forelegs was restricted, thus preventing grooming of the face, upper head and neck. Control animals were anaesthetized but were not wrapped. After recovering from the anaesthesia control rats groomed the face, head and abdomen. Partial-restraint stress was always performed between 1000 and 1200 h.

Rectal distension procedure

To prevent recording artefacts owing to movement during distension, rats were accustomed, three days before distension, to being placed in a polypropylene tube (diam. 6 cm, length 22 cm). A balloon consisting of an arterial embolectomy catheter (Fogarty, Edwards Laboratories, Santa Anna, USA) was introduced into the rectum 1 cm from the anus and fixed at the base of the tail. The balloon (diam. 2 mm, length 2 cm) was increasingly inflated with 0·4 mL water, starting from 0·4 mL up to 1·2 mL. Each inflation step lasted 5 min. The choice of a 5 min duration for each inflation was based on technical considerations because 5 min is the normal duration enabling relevant measurement of abdominal cramp (Morteau et al 1994a). At the end of the distension, the water was withdrawn to enable detection of leakage.

Experimental protocol

In a first series of experiments performed on seven groups of eight rats, rectal distension was performed 1 day before and 3 days after intracolonic instillation of trinitrobenzenesulphonic acid. Ten minutes before rectal distension the animals were treated intraperitoneally with 0·5 mL vehicle (dimethylsulphoxide and water, 50 : 50 v/v) containing trimebutine or *N*-desmethyltrimebutine at doses of 5, 10 or 20 mg kg^{-1}.

In a second series of experiments, seven groups of 8–12 rats were submitted to rectal distension 2 h before and 30 min after a 2-h restraint-stress session. In this series of experiments the animals were

also treated 10 min before rectal distension with trimebutine or desmethyltrimebutine at the same doses as in the first series of experiments.

In a third series of experiments performed on three additional groups of rats, naloxone ($1\,mg\,kg^{-1}$) or vehicle (0·9% NaCl) was injected subcutaneously 10 min before trimebutine or *N*-desmethyltrimebutine.

Data analysis

Statistical analysis of the number of abdominal spike bursts occurring during each 5-min period was performed by Dunnett's procedure for multiple comparisons, after analysis of variance. $P < 0.05$ was considered as indicative of statistical significance. All the values are expressed as means ± s.e.m.

Results

Trinitrobenzenesulphonic acid-induced rectal hyperalgesia

Effects of trimebutine and desmethyltrimebutine on abdominal response to rectal distension. Before treatment with trinitrobenzenesulphonic acid, rectal distension progressively increased the number of abdominal cramps; this increase was significant for rectal distension volumes between 0·8 and 1·6 mL (Table 1, Figure 1). Intraperitoneal trimebutine at doses of 10 and $20\,mg\,kg^{-1}$ significantly ($P < 0.05$) reduced the number of abdominal cramps observed for the highest volume of distension, 1·6 mL, but also for 1·2-mL distension at a dose of $20\,mg\,kg^{-1}$. At the lowest dose ($5\,mg\,kg^{-1}$), trimebutine did not affect the number of abdominal cramps regardless of distension volume (Table 1).

Injected 30 min before rectal distension, desmethyltrimebutine did not affect the abdominal cramps generated by rectal distension regardless of dose (5, 10 and $20\,mg\,kg^{-1}$) or the distension volume (Table 1, Figure 1).

Effects of trimebutine and desmethyltrimebutine on trinitrobenzenesulphonic acid-induced hyperalgesia. Compared with values observed before inflammation, trinitrobenzenesulphonic acid significantly ($P < 0.05$) increased the number of abdominal cramps generated by 0·4, 0·8 and 1·2-mL rectal distension (Figure 1, Table 2).

Injected before rectal distension, doses of trimebutine as low as $5\,mg\,kg^{-1}$ attenuated this increase for a distension volume of 0·8 mL; doses of 10 and $20\,mg\,kg^{-1}$ suppressed abdominal cramps generated by all distension volumes (Figure 1, Table 2). In contrast, doses of desmethyltrimebutine from 5 to $20\,mg\,kg^{-1}$ had no significant effect on the trinitrobenzenesulphonic acid-induced increase in the abdominal response to distension (Table 2). Naloxone, previously administered subcutaneously at a dose of $1\,mg\,kg^{-1}$, suppressed the effects of trimebutine on the trinitrobenzenesulphonic acid-induced increase in abdominal response to rectal distension (Table 3).

Stress-induced rectal hyperalgesia

Effect of partial-restraint stress. Rectal distension applied in control rats significantly and gradually increased the number of abdominal discharges from a threshold volume of 0·8 mL, in a manner similar to that observed in the first series of experiments. Partial-restraint stress applied for 2 h reduced to 0·4 mL the threshold of abdominal response to rectal distension and significantly ($P < 0.05$) increased, by 76·8 and 41·6%, the number of abdominal contractions observed for 0·8- and 1·2-mL rectal distension (Figure 2).

Effects of trimebutine and desmethyltrimebutine. When administered at a dose of $5\,mg\,kg^{-1}$ 10 min before rectal distension and after partial-restraint stress, trimebutine did not modify the stress-induced increase in abdominal response to rectal

Table 1. Effect of intraperitoneal trimebutine and *N*-desmethyltrimebutine on the number of abdominal spike bursts generated by increasing volumes of rectal distension in rats.

Treatment	Dose ($mg\,kg^{-1}$)	Abdominal cramps/15 min Distension volume (mL)				
		0	0·4	0·8	1·2	1·6
Vehicle	–	1·2 ± 0·4	6·7 ± 1·7	17·6 ± 2·3	26·9 ± 2·7	30·3 ± 1·8
Trimebutine	5	0·9 ± 0·6	5·8 ± 0·7	18·1 ± 2·5	24·7 ± 2·3	30·2 ± 1·7
	10	1·3 ± 0·5	5·9 ± 0·6	17·1 ± 1·9	24·8 ± 2·2	26·1 ± 1·9*
	20	0·8 ± 0·7	5·2 ± 1·2	17·2 ± 0·9	21·4 ± 2·3*	23·1 ± 2·1*
N-Desmethyltrimebutine	5	0·8 ± 0·1	4·7 ± 1·1	19·6 ± 2·1	25·1 ± 2·6	27·5 ± 3·3
	10	1·1 ± 0·4	6·7 ± 1·5	18·1 ± 1·3	26·6 ± 1·6	29·3 ± 1·8
	20	1·9 ± 0·7	5·3 ± 1·5	13·7 ± 2·7	28·4 ± 2·0	28·7 ± 2·1

Results are means ± s.e.m. (n = 8). *$P < 0.05$, significantly different from corresponding results for vehicle.

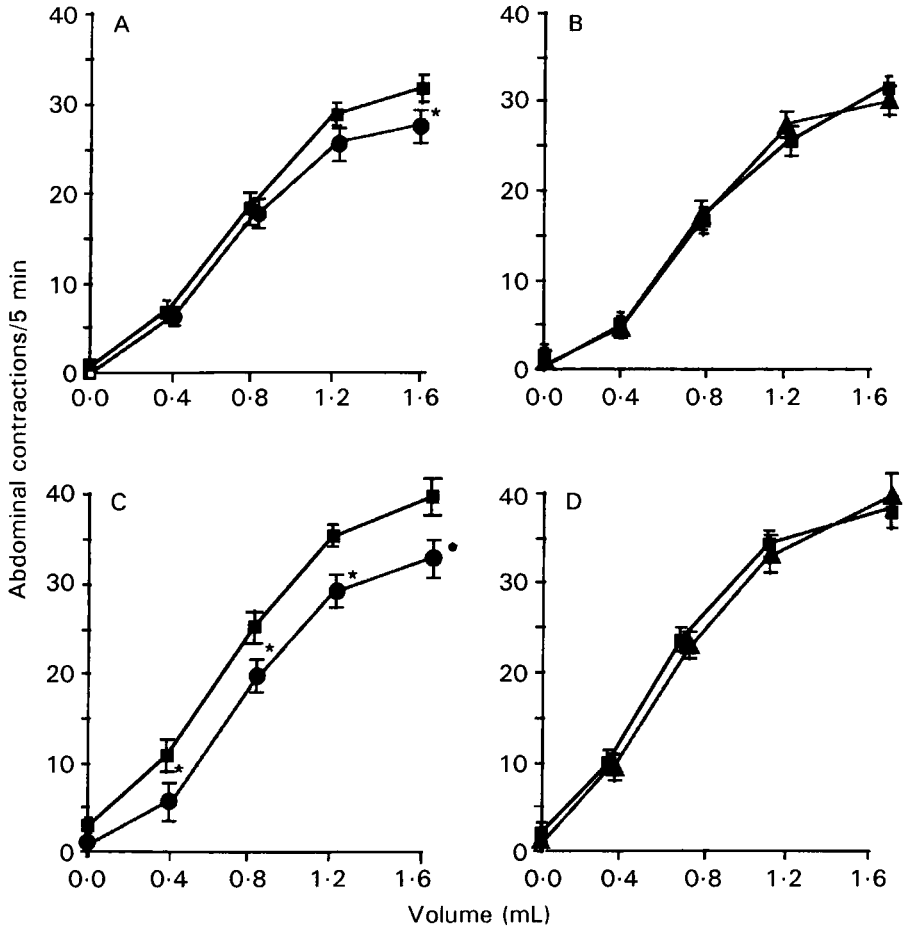

Figure 1. Influence of trimebutine (●) and *N*-desmethyltrimebutine (▲) at intraperitoneal doses of $10 mg kg^{-1}$, compared with vehicle (■), on the number of abdominal contractions induced by step rectal distension before (A, B) and 3 days after (C, D) rectal instillation with trinitrobenzenesulphonic acid. Results are means ± s.e.m. (n = 8). $*P < 0.05$, significantly different from corresponding results for vehicle.

Table 2. Effect of trimebutine and *N*-desmethyltrimebutine on rectocolitis-induced increase in the number of abdominal cramps induced by 0·8-mL rectal distension in rats.

Treatment	Dose ($mg kg^{-1}$)	Abdominal contractions/5 min
Vehicle		6·3 ± 0·2
Trimebutine	5	3·2 ± 0·18*
	10	0·4 ± 0·6**
	20	0·3 ± 0·5**
Vehicle		5·9 ± 1·6
N-Desmethyltrimebutine	5	4·8 ± 1·4
	10	4·7 ± 0·9
	20	3·9 ± 1·5

Results are means ± s.e.m. (n = 8). $*P < 0.05$, $**P < 0.01$, significantly different from corresponding results for vehicle.

distension. In contrast, 10 and $20 mg kg^{-1}$ trimebutine reduced and suppressed, respectively, stress-induced hyperalgesia regardless of distension volume (Figure 2).

When injected intraperitoneally 20 min after the stress session at doses as low as $5 mg kg^{-1}$, desmethyltrimebutine reduced by 56·2% the stress-induced enhancement of abdominal spike discharges observed for 1·2-mL distension. At higher doses (10 and $20 mg kg^{-1}$) desmethyltrimebutine abolished the effects of stress on rectal sensitivity (Figure 2).

Antagonism by naloxone

Injected subcutaneously 20 min before rectal distension, naloxone ($1 mg kg^{-1}$) did not significantly affect the number of abdominal cramps in control animals or stress-induced enhancement of abdominal response. However, when injected 10 min before trimebutine ($10 mg kg^{-1}$) applied 3 days after trinitrobenzenesulphonic acid, naloxone reversed the effects of trimebutine on inflammation-induced hyperalgesia (Table 3, 4). At the same dose naloxone did not affect the effects of trimebutine ($10 mg kg^{-1}$) on partial-restraint-stress-induced hyperalgesia for a distension volume of 1·2 mL. Furthermore, naloxone ($1 mg kg^{-1}$) seemed unable to attenuate significantly the antinociceptive

Table 3. Effect of intraperitoneal trimebutine and *N*-desmethyltrimebutine on the number of abdominal cramps induced by rectal distension after restraint stress in rats.

Treatment	Dose ($mg\,kg^{-1}$)	Abdominal contractions/5 min Distension volume (mL)			
		0	0·4	0·8	1·2
Vehicle	–	0·8 ± 0·5	6·1 ± 0·6	17·9 ± 1·8	25·7 ± 1·4
Trimebutine	5	1·2 ± 0·7	6·4 ± 0·9	18·2 ± 2·1	25·1 ± 2·2
	10	1·1 ± 0·3	6·2 ± 1·5	14·6 ± 2·2	21·5 ± 1·8*
	20	0·9 ± 0·7	7·0 ± 0·7	12·2 ± 1·7*	17·6 ± 1·8*
Vehicle	–	0·8 ± 0·5	6·8 ± 1·1	20·5 ± 1·8	27·4 ± 1·7
N-Desmethyltrimebutine	5	1·4 ± 0·4	6·2 ± 1·3	15·5 ± 1·8	20·9 ± 1·4*
	10	0·9 ± 1·1	6·3 ± 1·9	11·6 ± 1·7*	16·5 ± 1·0*
	20	1·2 ± 1·0	6·3 ± 1·4	12·0 ± 2·2*	17·3 ± 2·6*

Results are means ± s.e.m. (n = 8). *$P < 0.05$, significantly different from corresponding results for vehicle.

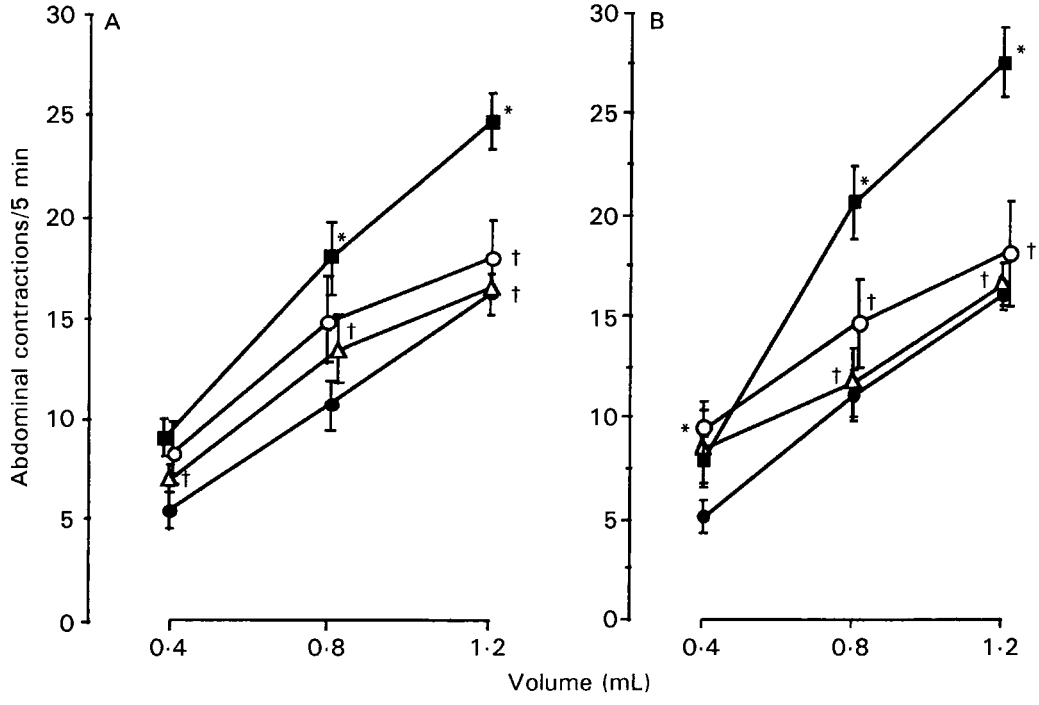

Figure 2. Effects of increasing doses of trimebutine (A) and *N*-desmethyltrimebutine (B) on the restraint-stress-induced increase in abdominal cramps in response to rectal distension in rats. ●, Control; ■, stress + vehicle; △, stress + $10\,mg\,kg^{-1}$ drug; ○, stress + $20\,mg\,kg^{-1}$ drug. Results are means ± s.e.m. (n = 8). *$P < 0.05$, significantly different from corresponding results for vehicle. †$P < 0.05$, significantly different from corresponding results for stress + vehicle.

effects of desmethyltrimebutine on the stress-induced increase in abdominal spike activity in response to rectal distension (Table 3, 4).

Discussion

Our results demonstrate that trimebutine and its major metabolite, desmethyltrimebutine, affect rectal barosensitivity in rats differently, depending upon the basal state. Moreover these drugs seem selectively more active against rectal hyperalgesia induced by local inflammation and stress than on basal sensitivity, these effects being only partly related to action on opiate receptors.

As previously described (Morteau et al 1994a), the induction of rectocolitis by trinitrobenzenesulphonic acid in ethanol evokes an increase in the sensitivity to rectal distension by reducing the threshold of appearance of abdominal cramps and by enhancing the response to rectal distension, an effect unrelated to alterations in rectal compliance. This hypersensitivity induced by locally applied trinitrobenzenesulphonic acid is reduced by 5-HT_3 receptor antagonists but there is no clear evidence

Table 4. Antagonism by naloxone of the effects of trimebutine and *N*-desmethyltrimebutine on inflammation induced by trinitrobenzenesulphonic acid and the stress-induced increase in abdominal response to rectal distension in rats.

	Abdominal contractions/5 min		
	After trinitrobenzene sulphonic acid Distension volume (mL)		After stess Distension volume (mL)
	0·4	1·6	1·2
Vehicle (0·9% NaCl)	6·1 ± 1·2	9·3 ± 0·7	7·0 ± 1·3
Trimebutine (10 mg kg^{-1}, i.p.)	1·7 ± 0·5*	2·6 ± 0·2*	3·2 ± 1·4*
Naloxone (0·3 mg kg^{-1}, s.c.)	6·2 ± 1·4	8·5 ± 0·7	6·4 ± 1·2
Trimebutine + naloxone (1 mg kg^{-1})	6·0 ± ·08	9·0 ± 0·8	2·0 ± 0·2*
Vehicle (0·9% NaCl)		9·3 ± 0·7	9·2 ± 0·6
N-Desmethyltrimebutine (10 mg kg^{-1}, i.p.)		2·6 ± 0·2	2·0 ± 0·8*
Naloxone (0·3 mg kg^{-1}, s.c)		8·5 ± 0·7	8·5 ± 0·7
N-Desmethyltrimebutine + naloxone (1 mg kg^{-1})		9·0 ± 0·8	2·3 ± 1·5*

Results are means ± s.e.m. (n = 8). *$P < 0.05$, significantly different from corresponding results for vehicle.

that the involvement of 5-HT$_3$ receptors is selective for inflammation-induced hyperalgesia (Morteau et al 1994b). In contrast, mediators such as bradykinin seem to be selectively involved in triggering trinitrobenzenesulphonic acid-induced hyperalgesia because a B_2 antagonist (HOE140) is active on rectal nociception only after induction of rectocolitis (Julia et al 1995).

Trimebutine, a drug extensively used for treatment of irritable bowel syndrome has peripheral opioid agonist properties related to affinities for μ and κ opioid receptor subtypes (Pascaud et al 1989). Several studies indicate that opioid agonists might have peripherally mediated antinociceptive properties (Smith & Wilkinson 1982). It has also been shown that selective κ agonists such as U50488 and fedotozine act peripherally to inhibit nociceptive inputs generated by colorectal distension with potent visceral antinociceptive efficacy on hyperalgesia resulting from inflammatory reactions (Langlois et al 1997). Our current studies have shown that trimebutine is four to ten times more potent at reducing abdominal cramps related to rectal distension after trinitrobenzenesulphonic acid-induced rectal inflammation and that this effect is blocked by naloxone, suggesting that it is linked to an activation of opiate receptors.

Recently, pharmacological and electrophysiological data have contributed to the assessment of the peripheral analgesic effects of opiates-μ and κ agonists have been found to block nociception efficiently when given at the site of irritation at doses that are not active systemically (Stein 1993). These effects are blocked by antagonists such as quaternary alkaloids, which penetrate the blood–brain barrier poorly (Smith & Wilkinson 1982), and higher antinociceptive effects of these compounds are found when they are administered locally at the site of inflammation (Stein 1993). Accordingly, the receptors involved in the peripheral mechanism of action of opioids are probably located on the very distal ends of primary afferent neurones or on surrounding cells, an increased number of local opioid receptors being detected at the site of inflammation (Hassan et al 1993).

Partial-restraint stress increases the abdominal response to rectal distension, an effect which has previously been shown to be mediated by the CNS release of corticotrophin-releasing factor (CRF) (Gué et al 1997). In man, peripheral administration of CRF at doses inducing somatic analgesia (Hargreaves et al 1987) reduces the threshold and increases the intensity of sensation of discomfort to rectal distension (Lembo et al 1996). However, the mechanisms by which stress induces rectal hyperalgesia are not well understood. CRF might act by changing the excitability of dorsal horn neurons receiving converging inputs from different afferents (Mayer & Gebhart 1994) or is the consequence of an increase in the size of the receptive fields of dorsal horn neurons and spinal mechanisms (Cervero 1994). CRF can trigger the local release of proinflammatory mediators inducing sensitization of primary afferent endings (Schäfer et al 1997) and can also alter the brain-level processing of visceral sensory information as demonstrated for the locus coeruleus (Valentino et al 1992). Both trimebutine and its major metabolite desmethyltrimebutine are active on stress-induced rectal hyperalgesia at doses as low as 5 mg kg^{-1}, which are not active in the basal state. Unexpectedly, desmethyltrimebutine seems more active than trimebutine and its effects and those of trimebutine are not blocked by naloxone. Desmethyltrimebutine has a

lower affinity than trimebutine for μ and κ opioid receptors. Consequently we can speculate that these two compounds act centrally or peripherally on sensitization of terminals of primary afferents by interfering directly with other mechanisms and possibly by changes in ionic permeability (Roman et al 1998).

Although the causes and mechanisms of irritable bowel syndrome are not well understood, it is admitted that stress might contribute to visceral hypersensitivity because most patients with irritable bowel syndrome suffer from increased levels of anxiety and psychosocial distress (Drossman et al 1988). More recently it has been demonstrated that irritable bowel syndrome symptoms appear more frequently after severe gut-infectious diarrhoea in stressed patients suggesting that stress and gut inflammation act synergistically to trigger gut hyperalgesia (Gwee et al 1996).

Our experiments have shown that intraperitoneal trimebutine, at plasma concentrations equivalent to those obtained during classical treatment of oral irritable bowel syndrome, i.e. 300 to 600 mg twice a day, is active on hyperalgesia induced by both local inflammation and stress. However at this dose trimebutine might influence gut motility with a peripheral site of action (Valori & Shannon 1987) and these effects might also result from primary influence on gut afferents involved in functional reflexes.

Finally, whatever the mechanism involved trimebutine and its major metabolite desmethyltrimebutine are potent drugs inhibiting rectal hyperalgesia evoked by local inflammation and stress in rats and these effects might explain the efficacy of trimebutine in the treatment of functional bowel disorders.

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Nicotine-induced Perturbations on Heart Rate, Body Temperature and Locomotor Activity Daily Rhythms in Rats

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Abstract

The aim of this study was to evaluate the influence of nicotine on the daily rhythms of heart rate, body temperature and locomotor activity in unrestrained rats by use of implanted radiotelemetry transmitters.

The study was divided into three seven-day periods: a control period, a treatment period and a recovery period. The control period was used for baseline measurement of heart rate, body temperature and locomotor activity. During the treatment period three rats received nicotine (1 mg kg^{-1}, s.c.) at 0900 h. Three rats received saline under the same experimental conditions. Heart rate, body temperature and locomotor activity were continuously monitored and plotted every 10 min. During the three periods a power spectrum analysis was used to determine the dominant period of rhythmicity. If daily rhythms of heart rate, body temperature and locomotor activity were detected, the characteristics of these rhythms, i.e. the mesors, amplitudes and acrophases, were determined by cosinor analysis, expressed as means ± s.e.m. and compared by analysis of variance.

Nicotine did not suppress daily rhythmicity but induced decreases of amplitudes and phase-advances of acrophases for heart rate, body temperature and locomotor activity. These perturbations might result from the effects of nicotine on the suprachiasmatic nucleus, the hypothalamic clock that co-ordinates biological rhythms.

Nicotine is known to perturbate some physiological parameters such as heart rate, body temperature and locomotor activity in rats. Zarrindast et al (1995) demonstrated that nicotine injections (0·5, 1 and 2 mg kg^{-1}) induced dose-dependent hypothermia for 15 min after drug administration. Nicotine injections at different doses (6·25 μg kg^{-1} min^{-1}, 12·5 μg kg^{-1} min^{-1} and 25 μg kg^{-1} min^{-1}) in drug-naive and in chronic smoke-exposed rats induced tachycardia which increased during the first 15 min after drug administration (Barron et al 1988). Kita et al (1986) studied nicotine-induced variations of ambulatory activity in rats for 120 min after nicotine injection and demonstrated that during the light period a large dose (0·5 mg kg^{-1}) of nicotine induced a stimulant effect during the first 20 min after injection whereas after injection during the dark period the same dose of nicotine induced ataxia. A small dose (0·1 mg kg^{-1}) induced only ataxia during the first 20 min after injection during the dark period.

Nevertheless, most work has focused on the period immediately after nicotine injections and to the best of our knowledge the effects of nicotine on the daily rhythms of well-known markers such as body temperature, heart rate and locomotor activity have not been evaluated over a 24-h period. To clarify the longer-term effects of nicotine upon daily rhythms we have investigated the possible modifications induced by repeated injection of nicotine on the daily rhythms of temperature, heart rate and locomotor activity in rats and compared them with those of controls receiving saline. Such a protocol takes into account the possibility of stress-induced effects arising from handling or injection.

Materials and Methods

Animals and housing

For a minimum of three weeks before use six Wistar AF IOPS adult male rats from Iffa-Credo (St Germain-sur-l'Arbresle, France), mean weight 275 g, ten-weeks-old, were housed in individual

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transparent polypropylene cages (40 × 30 × 30 cm) under controlled environmental conditions—relative humidity (50–55%), temperature (24 ± 1°C) and synchronized by a 12-h light-dark cycle (light on from 0600 h to 1800 h). Lighting was provided by two 60-W fluorescent tubes and lighting intensity was approximately 300 lx at cage level. Food and water were freely available and changes occurred once a week on an irregular schedule. The experiments were conducted in accordance with internationally accepted principles concerning the care and use of laboratory animals (National Research Council 1985) after acceptance by our animal experimentation ethical committee (Commission Consultative d'Ethique Animale, Centre de Formation et de Recherches Expérimentales Médico-Chirurgicales, Faculté de Médecine de Marseille, France).

Experimental procedures

Heart rate, body temperature and locomotor activity were measured by radiotelemetry. Just before implantation calibration values for each transmitter were entered into the Dataquest III data-acquisition system. Surgical implantation of the transmitters (model TA11-CTA-F40, Data Sciences, St Paul, MN), as described by Kramer et al (1993), was performed under ketamine hydrochloride (100 mg kg^{-1} i.p.) general anaesthesia. Shortly after full anaesthesia was achieved a 2-cm incision was made in the peritoneum and the sensor was implanted into the abdominal cavity and sutured to the abdominal wall. ECG leads were extended subcutaneously to the right axilla and to the left lower rib area and sutured to muscle tissue. After closure animals were monitored for 2 h until they recovered from anaesthesia; they were then returned to their home cage and recording was started. Signals from the transmitter were received by an antenna mounted in a receiver board (model CTR86, Data Sciences) placed under the animal's cage. Data of heart rate (beats min^{-1}), temperature (°C) and locomotor activity (counts) were collected as described by Meerlo et al (1995) every 10 min over a 21-day period and processed by means of a PC with a specialized recording and analysis system (Dataquest III, Data Sciences).

Drugs

Nicotine (Sigma, France) was dissolved in saline (0·9%) and injected subcutaneously at 1 mg kg^{-1}, a dose considered to be pharmacologically active and representing approximately 1/30th the LD50 (dose resulting in the death of half of the animals) in rats (Zarrindast et al 1995). The animals of the control group were injected with saline under the same experimental conditions.

Protocol

After a period of recovery from surgical implantation and anaesthesia (Prudian et al 1997), the study was divided into three seven-day periods. The first week was a control period for baseline measurement of daily rhythms in heart rate, body temperature and locomotor activity. This period was characterized by daily handling and weighing of the animals (0900 h) only. The second week was the treatment period—three animals (nicotine group) received daily nicotine at 0900 h and three animals (control group) received saline at the same time. The third week was a recovery period.

Data analysis

Heart rate, body temperature and locomotor activity were measured every 10 min and analysed by two methods by use of the Dataquest III data-acquisition system. Firstly, to determine the dominant period during the control, treatment and recovery periods a power spectrum analysis (Fourier transform) was applied to 30-min average data intervals. Then, to assess daily variations of heart rate, body temperature and locomotor activity, least-square cosine regression (cosinor analysis) was applied to individual data for the control, treatment and recovery periods and a rhythm was considered to be significantly detected when $P < 0{\cdot}05$ (Refinetti 1992). The daily rhythm characteristics of heart rate, body temperature and locomotor activity, i.e. mesor (midline estimating statistic of rhythm corresponding to the mean level, which is equal to the 24-h average), amplitude (half of the peak-to-trough difference of the fitted cosine function) and acrophase (the crest time of rhythm given in degrees (°), where 360° correspond to a 24-h cycle and the starting time of 0000 h was denoted by 0°) were estimated by the linear method of least squares (Morgan & Minors 1995) and expressed as means ± s.e.m.

Three-way analysis of variance was used for statistical analysis (Statview II program) using the three factors period (control period, treatment period or recovery period), the day of the period (day 1–7) and the treatment (nicotine or control). If no interaction related to the days within a period was detected, comparisons between control, treatment and recovery periods for nicotine and control groups and comparisons between nicotine and control groups for the control, treatment and recovery periods were performed by one-way analysis of variance; if a significant difference was found, Fisher's PLSD (protected last significant difference) test for multiple comparisons was applied.

Results

Fourier analysis

Fourier analysis showed that the daily rhythms of heart rate, body temperature and locomotor activity were significantly validated for each rat during each time-span of the protocol, i.e. each rhythm had a dominant period of 24 h.

Statistical analysis of cosinor parameters

Because Fourier analysis showed that all the rhythms were significantly detected for 24 h, cosinor analysis with a 24-h period was used to determine the characteristics of these rhythms, i.e. the mesors, amplitudes and acrophases. These parameters were expressed as means ± s.e.m. and compared by analysis of variance. As the three-way analysis of variance did not detect any interaction related to the day (day 1–7) and the period of the protocol (control, treatment and recovery periods), one-way analysis of variance was performed to compare: mesors, amplitudes and acrophases of the nicotine and control groups during the three periods of the protocol; and mesors, amplitudes and acrophases between the nicotine and control groups for each period of the protocol.

Tables 1–3 show the mean values of the mesors, amplitudes and acrophases for daily rhythms of heart rate, body temperature and locomotor activity, respectively.

Whatever the period of the protocol (control, treatment or recovery), mesor values of heart rate, body temperature and locomotor activity did not change for the control or nicotine-treated groups. Although the amplitudes of heart rate, body temperature and locomotor activity for both groups were significantly reduced during the treatment period in comparison with the control and recovery periods, the observed decreases were significantly ($P < 0.05$) more pronounced for the nicotine group than for the control group. Acrophases of heart rate, body temperature and locomotor activity were phase-advanced during the treatment period compared with the control and recovery periods, unlike the controls, for which acrophases were unmodified whatever the period.

Figures 1–3 show the mesors, amplitudes and acrophases of heart rate, body temperature and locomotor activity, respectively, for the nicotine and control groups over the three seven-day periods of the protocol. These data are expressed as means ± s.e.m.

Table 1. Mesor, amplitude and acrophase of heart rate daily rhythm for nicotine and control groups for each period of the protocol.

	Mesor (beats min^{-1})		Amplitude (beats min^{-1})		Acrophase (°)	
	Nicotine	Control	Nicotine	Control	Nicotine	Control
Control	341·29 ± 4·12	336·41 ± 3·09	44·48 ± 1·76	45·97 ± 2·10	356·56 ± 2·13	357·99 ± 1·65
Treatment	344·23 ± 3·89	338·06 ± 2·54	25·96 ± 1·74*‡	35·80 ± 2·08*	348·09 ± 2·39*‡	354·76 ± 2·41
Recovery	346·65 ± 5·80	338·06 ± 1·60	41·76 ± 1·89†	41·82 ± 2·35†	356·14 ± 1·66†	355·05 ± 1·92
Analysis of variance	$P = 0.6297$, $F = 1.056$		$P = 0.0001$, $F = 17.968$		$P = 0.0123$, $F = 3.094$	

Values are mean ± s.e.m. Results were compared by one-way analysis of variance. If a statistically significant difference was detected, post-hoc comparisons were performed by Fisher's PLSD test. *$P < 0.05$, significantly different from result for control period; †$P < 0.05$, significantly different from result for treatment period; ‡$P < 0.05$, significantly different from control result for the same period.

Table 2. Mesor, amplitude and acrophase of body temperature daily rhythm for nicotine and control groups for each period of the protocol.

	Mesor (°C)		Amplitude (°C)		Acrophase (°)	
	Nicotine	Control	Nicotine	Control	Nicotine	Control
Control	37·69 ± 0·03	37·67 ± 0·03	0·60 ± 0·03	0·60 ± 0·03	361·70 ± 2·38	361·51 ± 2·16
Treatment	37·77 ± 0·03	37·67 ± 0·03	0·36 ± 0·02*‡	0·45 ± 0·02*	347·68 ± 2·09*‡	361·53 ± 4·12
Recovery	37·68 ± 0·03	37·66 ± 0·04	0·62 ± 0·02†	0·59 ± 0·04†	354·36 ± 1·45†	357·66 ± 2·28
Analysis of variance	$P = 0.0654$, $F = 2.1388$		$P = 0.0001$, $F = 14.075$		$P = 0.0001$, $F = 17.342$	

Values are mean ± s.e.m. Results were compared by one-way analysis of variance. If a statistically significant difference was detected, post-hoc comparisons were performed by Fisher's PLSD test. *$P < 0.05$, significantly different from result for control period; †$P < 0.05$, significantly different from result for treatment period; ‡$P < 0.05$, significantly different from control result for the same period.

Table 3. Mesor, amplitude and acrophase of locomotor activity daily rhythm for nicotine and control groups for each period of the protocol.

	Mesor (Counts)		Amplitude (Counts)		Acrophase (°)	
	Nicotine	Control	Nicotine	Control	Nicotine	Control
Control	36·1 ± 1·9	38·4 ± 1·6	23·75 ± 1·09	28·61 ± 1·86	372·30 ± 4·31	378·39 ± 3·52
Treatment	36·8 ± 1·9	35·8 ± 1·8	12·48 ± 1·19*‡	19·64 ± 1·29*	347·83 ± 4·60*‡	389·53 ± 4·23
Recovery	34·1 ± 1·1	38·4 ± 2·5	21·55 ± 2·38†	27·63 ± 2·14†	374·96 ± 3·68†	387·26 ± 7·43
Analysis of variance	$P = 0·145$, $F = 1·6857$		$P = 0·0001$, $F = 11·832$		$P = 0·005$, $F = 3·59$	

Values are mean ± s.e.m. Results were compared by one-way analysis of variance. If a statistically significant difference was detected, post-hoc comparisons were performed by Fisher's PLSD test. * $P < 0·05$, significantly different from result for control period; † $P < 0·05$, significantly different from result for treatment period; ‡ $P < 0·05$, significantly different from control result for the same period.

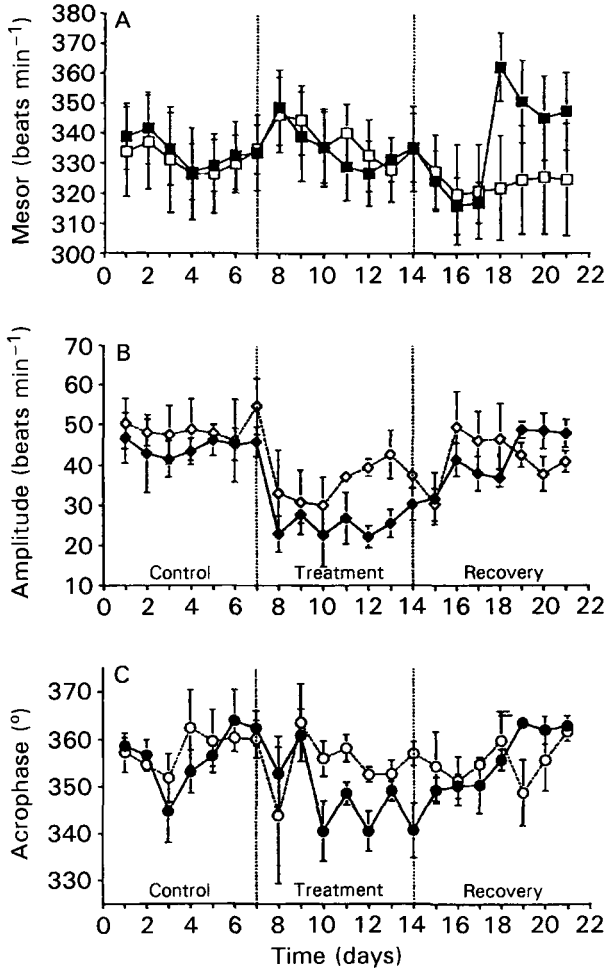

Figure 1. Mesor (A), amplitude (B) and acrophase (C) of heart rate for the nicotine (■, ◆, ●) and control (□, ◇, ○) groups over the 21-day control, treatment and recovery periods of the protocol. Each point represents the mean ± s.e.m. for the 24-h period for the three rats of each group.

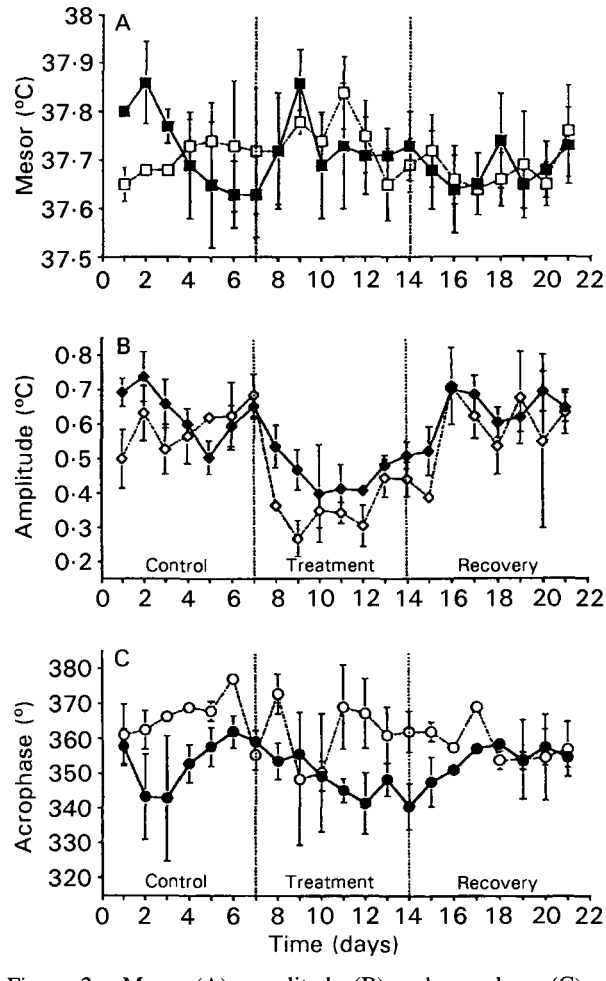

Figure 2. Mesor (A), amplitude (B) and acrophase (C) of body temperature for the nicotine (■, ◆, ●) and control (□, ◇, ○) groups over the 21-day control, treatment and recovery periods of the protocol. Each point represents the mean ± s.e.m. for the 24-h period for the three rats of each group.

Discussion

This study has demonstrated that: repeated administration of nicotine did not suppress the daily rhythmicity of heart rate, body temperature and locomotor activity; repeated administration of nicotine modified the amplitudes and acrophases of the three rhythms; and the modifications of the amplitudes were significantly more pronounced for the nicotine group than for the control group and the phase-shifts of acrophases, described during the

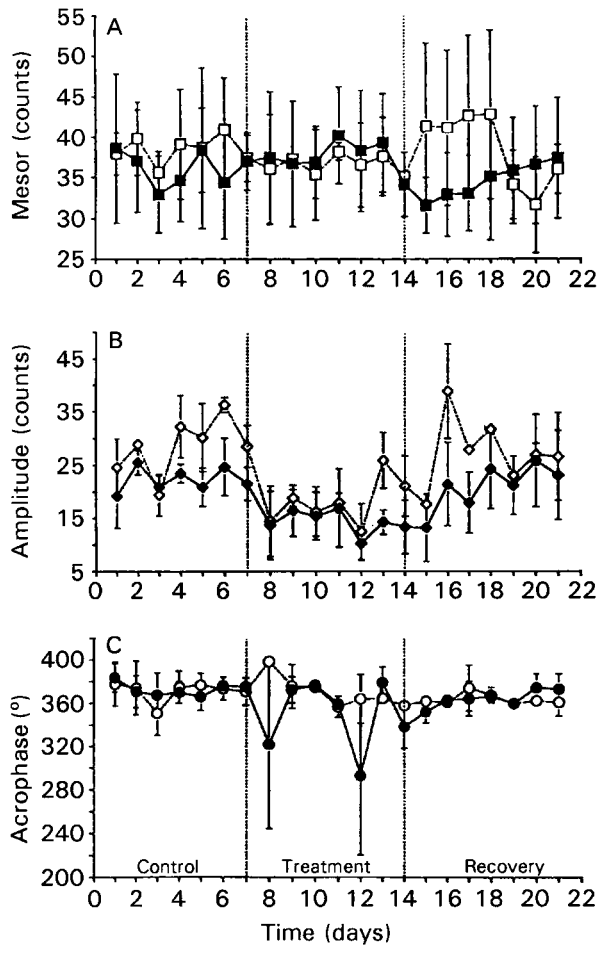

Figure 3. Mesor (A), amplitude (B) and acrophase (C) of locomotor activity for the nicotine (■, ◆, ●) and control (□, ◇, ○) groups over the 21-day control, treatment and recovery periods of the protocol. Each point represents the mean ± s.e.m. for the 24-h period for the three rats of each group.

treatment period, were only observed for the nicotine group.

These data confirmed that nicotine perturbated the daily rhythms of heart rate, body temperature and locomotor activity. The statistical differences between the two groups indicated that the effect of nicotine was genuine. Furthermore, it was of interest to show that the stress induced by saline injections significantly modified the amplitudes of the three rhythms, in agreement with previous reports of the modification of such rhythms induced by different kinds of stress (Harper et al 1996).

In rodents physiological and behavioural rhythms are co-ordinated by the suprachiasmatic nucleus (Mirmiran et al 1995; Sano et al 1995). In-vivo observations have revealed that the cholinergic system is involved in the regulation of these rhythms. Carbachol, a non-specific cholinergic agonist, phase-shifts circadian activity rhythms in rodents (Bina & Rusak 1996) and Trachsel et al (1995) demonstrated that nicotine phase-advanced the circadian neuronal activity rhythms in-vitro in rat suprachiasmatic nuclei explants. Our results were in total agreement with these observations and must be completed with further studies to determine the nicotinic receptors involved in the effects.

Another hypothesis should be explored. Dopamine is involved in the central effects of nicotine (Benwell & Balfour 1997) and Marshall et al (1997) reported that nicotine elicited a dose-dependent increase in dopamine release in the striatum and in the nucleus accumbens. As described by Zarrindast et al (1995), nicotine-induced modification of body temperature might be mediated by an indirect dopaminergic mechanism. Fung et al (1996) observed a significant decrease of locomotor activity and a reduction of the dopamine content of the nucleus accumbens in rats after the cessation of 14-day nicotine administration. According to Taylor (1996), the nicotine-induced increases of heart rate and blood pressure in mammals were a result of stimulation of the sympathetic ganglia together with the discharge of monoamines from sympathetic nerve endings. All these data, even if obtained under experimental conditions different from those used in our study, clearly indicated that dopamine participated in the effects of nicotine on the regulation of heart rate, body temperature and locomotor activity in rats. Smith et al (1992) reported that dopamine concentrations followed a daily rhythm and Pietilä et al (1995) showed that chronic oral administration of nicotine affected the circadian rhythm of dopamine in the striata of mice. These findings suggest that repeated administration of nicotine altered the circadian pattern of striatal dopamine and thus might affect the functions regulated by these transmitters.

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Influence of Twinline, an Elemental Diet, on the Generation of Nitric Oxide and Reactive-oxygen Intermediates from Mouse Peritoneal Macrophages and Polymorphonuclear Leukocytes

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Abstract

The influence of Twinline (SNN-6010), an elemental diet containing medium-chain triglycerides, on the generation of nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) has been examined in mouse peritoneal macrophages and polymorphonuclear leukocytes (PMN).

When PMN and peritoneal macrophages obtained from untreated mice were cultured in medium containing 0·1% and 1% (v/v) Twinline for 48 h and stimulated with phorbol myristate acetate or *N*-formyl-methionyl-leucyl-phenylalanine, their chemiluminescence and $O_2^{\cdot-}$ generation were strongly suppressed, as was NO generation from peritoneal macrophages. PMN and peritoneal macrophages obtained from mice fed Twinline for 30 days generated much smaller amounts of $O_2^{\cdot-}$ and NO compared with PMN and peritoneal macrophages from control mice. In conjunction with this suppressed NO generation, inducible NO synthase and its mRNA expression in peritoneal macrophages were suppressed by Twinline both in-vivo and ex-vivo. Although phagocytosis of PMN and peritoneal macrophages was not suppressed by Twinline; their candida-killing activity was markedly suppressed.

These results indicate that Twinline suppresses the host-defence function of PMN and peritoneal macrophages by down-regulating their generation of reactive-oxygen intermediates and NO.

Elemental diets have been widely used for post-operative and post-traumatic nutritional management and for the nutritional treatment of patients with advanced cancer and immunosuppressive infection (Moore & Moore 1991; Moss & Naylor 1994; Gogos & Kalfarontzos 1995; Campos et al 1996). Because cellular and humoral immune function is usually suppressed in debilitated patients, and malnutrition induces further suppression of cellular immunity (Harbige 1996; Krenitsky 1996), immunosuppression is inadvertently induced if inappropriate nutritional management is maintained for a long period. Although elemental diets should be free from immunosuppressive activity, the influence of such diets on the immune system, especially on the function of phagocytes, has rarely been investigated. Apart from immunosuppression, elemental diets might create circumstances conducive to bacteria and it has been reported that elemental diet induced overgrowth of Gram-negative enterococci and reduced secretion of bile and pancreatic juice into the intestine, resulting in an increase of mucous membrane-penetrating enterococci (Shou et al 1991; Deitch et al 1993; Xu et al 1993). The results of an animal study indicate that alimentation with elemental diet carries a potential risk of bacterial dissemination.

Infections in immunosuppressed patients are life-threatening. In the management of patients undergoing nutritional treatment with elemental diet, fungal infections are one of the most serious problems (Alden et al 1989). Macrophages and polymorphonuclear leukocytes (PMN) play an important role in the host defence against microbial invasion by phagocytosis and the release of reactive-oxygen intermediates, nitric oxide (NO), and proteolytic enzymes (Babior 1978; Ferrante 1989). It has already been demonstrated that the bactericidal and fungicidal activity of phagocytes depends largely on their generation of reactive-oxygen intermediates (Sasada et al 1987; Ferrante 1989), activity which is regulated by a variety of cytokines and NO (Moncada et al 1991; Cenci et al

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1993). Acting together with cytokines, NO up-regulates the microbicidal activity of PMN and the activity of macrophages (Denis & Gregg 1990; Summersgill et al 1992; Simms & D'Amico 1997).

Reactive-oxygen intermediates are bactericidal molecules which react with NO. The reactant peroxinitrite ($ONOO^-$), which is formed from superoxide ($O_2^{\cdot-}$) and NO, has very strong bactericidal activity (Zhu et al 1992). However, reactive-oxygen intermediates such as the hydroxy radical ($^{\cdot}OH$), $O_2^{\cdot-}$ and peroxinitrite have potent cell- and tissue-damaging action. For example, damage of the lung, cerebrum and kidney by reactive-oxygen intermediates has frequently been reported (Halliwell 1992; Evans 1993; Laskin & Pendino 1995; Baliga et al 1997; Shiraishi et al 1997; Shohami et al 1997).

Although this background illustrates the importance of investigating the influence of elemental diets on the function of macrophages and PMN, few studies have been conducted (Scheurlen et al 1989; Shou et al 1991, 1994). We have, therefore, investigated the influence of an elemental diet containing medium-chain triglycerides on the generation of reactive-oxygen intermediates and NO from mouse peritoneal macrophages and PMN.

Materials and Methods

Mouse peritoneal macrophages and PMN

Male CBA/J mice, 6 weeks, were fed a conventional solid diet (CRF-1; Oriental Yeast, Tokyo, Japan) or Twinline (SNN-6010; Otuka, Tokyo, Japan) (Table 1) for 15 or 30 days. On the 15th or 30th day thioglycolate medium (2·5%, 2 mL; Difco, Detroit, MI) was injected intraperitoneally. Five days after injection, peritoneal macrophages were collected by washing out the peritoneal cavity with phosphate-buffered saline (PBS; 5 mL) containing heparin (5 units mL^{-1}). The collected cells were left to adhere to plastic Petri dishes for 2 h, and adherent cells were removed from the dish surfaces with ethylenediaminetetraacetic acid (0·02% w/v). Heparin (0·1 units mL^{-1}) was added to blood obtained from the jugular vein and PMN were separated by means of Ficoll-Hypaque gradients. Contaminated red blood cells were removed by hypotonic shock. After washing with PBS the cells were suspended in RPMI 1640 containing foetal bovine serum (2% v/v). Purity and viability > 95% were confirmed by Giemsa staining and trypan blue exclusion, respectively.

Assay of $O_2^{\cdot-}$ generation

$O_2^{\cdot-}$ was assayed spectrophotometrically by a cytochrome c reduction method by means of a Shimadzu (Shimadzu Seisakusho, Kyoto, Japan) UV-300 double-wavelength spectrophotometer, equipped with a thermostatted (37°C) cuvette holder. Peritoneal macrophages or PMN (1×10^7 cells mL^{-1}) suspended in Hank's balanced salt solution and 100 μM cytochrome c (type VI; Sigma, St Louis, MO) were poured into each cuvette, adjusting to a final concentration of 1×10^6 cells mL^{-1}. The reaction mixtures in the cuvettes were pre-incubated at 37°C for 1 min, and a stimulating agent (50 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma) or 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma)) was added to the reaction mixtures. The

Table 1. Composition of Twinline.

Calories (kcal L^{-1})	1000
Protein (g L^{-1})	41·0
Fat (g L^{-1})	278·0
Vitamin A (Iunits L^{-1})	2070
Vitamin D (Iunits L^{-1})	135
Vitamin E (μmol L^{-1})	15·6
Vitamin B_1 (μmol L^{-1})	6·0
Vitamin B_2 (mmol L^{-1})	6·0
Vitamin B_6 (μmol L^{-1})	14·8
Vitamin B_{12} (nmol L^{-1})	2·4
Vitamin C (mmol L^{-1})	1·28
Pantothenic acid (μmol L^{-1})	42·9
Biotin (nmol L^{-1})	151·5
Folic acid (nmol L^{-1})	567
Ca (mmol L^{-1})	1·10
P (mmol L^{-1})	17·1
Mg (mmol L^{-1})	0·06
Na (mmol L^{-1})	28·8
K (mmol L^{-1})	30·2
Fe (mmol L^{-1})	0·11
Cu (μmol L^{-1})	3·6
Zn (μmol L^{-1})	145
Mn (μmol L^{-1})	29
Cl (mmol L^{-1})	30·2
Isoleucine (μmol L^{-1})	157·5
Leucine (μmol L^{-1})	274·8
Lysine (μmol L^{-1})	205·0
Methionine (μmol L^{-1})	119·4
Cystine (μmol L^{-1})	6·7
Phenylalanine(μmol L^{-1})	20·2
Tyrosine (μmol L^{-1})	73·8
Threonine (μmol L^{-1})	136·0
Tryptophan (μmol L^{-1})	27·8
Valine (μmol L^{-1})	214·3
Arginine (μmol L^{-1})	81·4
Histidine (μmol L^{-1})	75·7
Alanine (μmol L^{-1})	136·3
Asparagic acid (μmol L^{-1})	206·9
Glutamic acid (μmol L^{-1})	626·8
Glycine (μmol L^{-1})	97·1
Proline (μmol L^{-1})	270·8
Serine (μmol L^{-1})	208·1
Caprylic acid (mmol L^{-1})	131·4
Capric acid (mmol L^{-1})	1·8
Myristic acid (mmol L^{-1})	0
Palmitic acid (mmol L^{-1})	5·31
Stearic acid (mmol L^{-1})	3·0
Oleic acid (mmol L^{-1})	3·6
Linoleic acid (mmol L^{-1})	18·9
Linolenic acid (mmol L^{-1})	0
Other fatty acids (mg L^{-1})	0

kinetics of cytochrome c reduction were measured by measurement of absorbance change at 540–550 nm. The O_2^- concentration was calculated from the linear portion of the cytochrome c reduction curve using $\Delta\varepsilon_{550}$ cytochrome $c = 21{\cdot}1 \times 10^3$ mol L^{-1} cm^{-1}.

Chemiluminescence

Chemiluminescence was measured with a Jasco (Tokyo, Japan) CAF-100 calcium analyser. Cells suspended (5×10^5 cells mL^{-1}) in Hank's balanced salt solution containing 100 μM luminol were incubated for 1 min at 37°C, after which 50 ng mL^{-1} PMA or 10^{-7} M FMLP was added. Each activity was expressed as peak chemiluminescence intensity (mV).

NO (NO_2^-) determination

NO, quantified by accumulation of nitrite (NO_2^-) as a stable end-product, was determined by microplate assay (Cenci et al 1993). Briefly, pooled supernatant (100 μL) was incubated with an equal volume of Griess reagent (1% sulphanilamide, 0·1% *N*-1-naphthylethylenediamine dihydrochloride, 2·5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined with a Corona Electronic (Ibaraki, Japan) MTP-120 microplate reader. The nitrite concentration was calculated from a sodium nitrite standard curve.

Candida albicans

Sabouraud's broth medium (100 mL) was inoculated with *C. albicans* TIMM0134 (standard strain) and KSC1 (established from a patient with oral candidiasis) maintained on agar slants at 4°C, and then cultured for 24 h at 37°C. After three washes with pyrogen-free saline by centrifugation at 1500 *g* the cells in the yeast phase of growth were counted in a haemocytometer. They were resuspended in saline and the suspensions were then diluted to an appropriate concentration with saline. Viability was confirmed by plating serial dilutions on to Sabouraud's dextrose agar plates.

Phagocytosis

Peritoneal macrophages or PMN were co-incubated in Hanks' balanced salt solution with latex particles (diam. 1 μm) at a ratio of 1 : 100 for 1 h at 37°C. Phagocytized particles were studied by light microscopy. Peritoneal macrophages and PMN containing more than five particles were considered to be phagocytic cells. Phagocytic activity was expressed as the percentage of phagocytic cells in 400 cells observed.

Candida killing

Candida cells (TIMM0134 and KSC1) in the yeast phase of growth were labelled with ^{51}Cr for 1 h at 37°C at a concentration of 100 μCi/10^8 cells. After three washes candida cells and peritoneal macrophages or PMN in RPMI 1640 medium supplemented with 2% foetal bovine serum were mixed to give an effector/target ratio of 1 : 10 in a final volume of 0·2 mL/well in flat-bottomed 96-well plates. The plates were incubated for 4 h at 37°C, and the isotope activity of the supernatant (0·1 mL) from each well was counted by means of a gamma counter. The percentage cytotoxicity was calculated by use of the formula:

$$\%\ \text{cytotoxicity} = (\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release}) \times 100$$

where spontaneous release is the activity in target cells incubated without effectors, and maximum release is the activity in the supernatant after treatment of the candida cells with 0·1% (v/v) Triton X-100. All terms in the formula were expressed as counts min^{-1}.

Western blotting

Mouse peritoneal macrophages (2×10^6 cells mL^{-1}) were suspended in Hank's balanced salt solution and incubated at 37°C in the presence or absence of 0·1% Twinline. The reaction was terminated by adding ice-cold 15% trichloroacetic acid solution containing 2 mM phenylmethylsulphonyl fluoride and 1 mM sodium vanadate. The precipitate was washed with ice-cold ether–ethanol (1 : 1), dissolved in sodium dodecylsulphate (SDS) sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis (30 mA, 3 h), the proteins were transferred to Immobilon-P filters (Millipore), by use of a Sartorius semi-dry blotting apparatus, and incubated with anti-inducible NO synthase (iNOS) monoclonal antibody (Transduction Laboratories, Lexington, KY) for 40 min after 60-min incubation in 5% powdered skimmed milk at room temperature. Antibody was detected with peroxidase-conjugated rabbit anti-mouse IgG, and peroxidase-positive bands were detected by means of an ECL Western blotting detection system (Amersham).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from mouse peritoneal macrophages was isolated by standard procedures (Chomczynski & Sacchi 1987). The levels of iNOS-mRNA were

examined by the RT-PCR technique. Reverse transcription was performed at 42°C for 30 min and PCR was performed in a Perkin-Elmer (Vetterstetten, Germany) Cetus 480 thermal cycler. The cycle programme was: 30-s denaturation (94°C), 30-s annealing (60°C), and 90-s amplification (72°C) for 30 cycles with a final 2-min amplification. As a control, cDNA samples were amplified by use of β-actin primer. PCR samples electrophoresed on agarose gel were visualized by staining with ethidium bromide. Primers for iNOS and β-actin were purchased from Toyobo (Osaka, Japan). The primer sequences were: iNOS: sense 5′CCCTCCTCCCGAAGTTTCTGGCAGCAGC3′, antisense, 5′GGCTGTCAGAGCCTCGTGGCTTTGG3′; β-actin: sense, 5′ATCTGGCACCACACCTTCTACAAT GAGCTGCG3′, antisense, 5′CGTCATATCCTGCTTGCTGATCCACATCTGC3.

Statistical analysis

Results are expressed as means ± standard deviation (s.d.). Two mean values were compared with each other by use of Student's *t*-test; $P < 0.05$ was regarded as indicative of statistical significance.

Results

O_2^- generation and chemiluminescence

O_2^- generation from PMN and peritoneal macrophages obtained from Twinline-fed mice was slightly suppressed; significant suppression was observed when PMN and peritoneal macrophages obtained from mice fed Twinline for 30 days were stimulated with PMA (Figure 1A). In-vitro treatment of PMN and peritoneal macrophages with Twinline resulted in strong suppression of O_2^- generation (Figure 1B). Although 0·01% (v/v) Twinline led to slight suppression of O_2^- generation, 0·1% Twinline suppressed O_2^- generation to about two-thirds of the control level ($P < 0.05$ or 0·01) and 1% Twinline caused a suppression to about half the control level ($P < 0.01$ or 0·001).

Chemiluminescence was suppressed by in-vivo and ex-vivo treatment of PMN and peritoneal macrophages with Twinline (Table 2). The amount of the suppression was slight after 15-day Twinline feeding and with 0·01% Twinline in-vitro treatment, but strong suppression of chemiluminescence was observed after 30-day Twinline feeding and in-vitro treatment with 1% Twinline.

Phagocytosis and candida killing

Although a slightly lower amount of phagocytic cells was observed in peritoneal macrophages treated with 1% Twinline than in control peritoneal macrophages, phagocytosis of peritoneal macrophages was not suppressed by in-vivo treatment with Twinline (Table 3), neither was the phagocytosis of PMN suppressed by either in-vivo or in-vitro treatment.

The influence of Twinline on the candida-killing activity of PMN and peritoneal macrophages was similar to that on O_2^- generation (Figures 2A, B). Compared with the suppression of killing by in-vivo Twinline, the suppression of candida killing by 1% Twinline was greater. In both candida cell lines the fungicidal activity of 1% Twinline-treated PMN and peritoneal macrophages was reduced to approximately half of control activity.

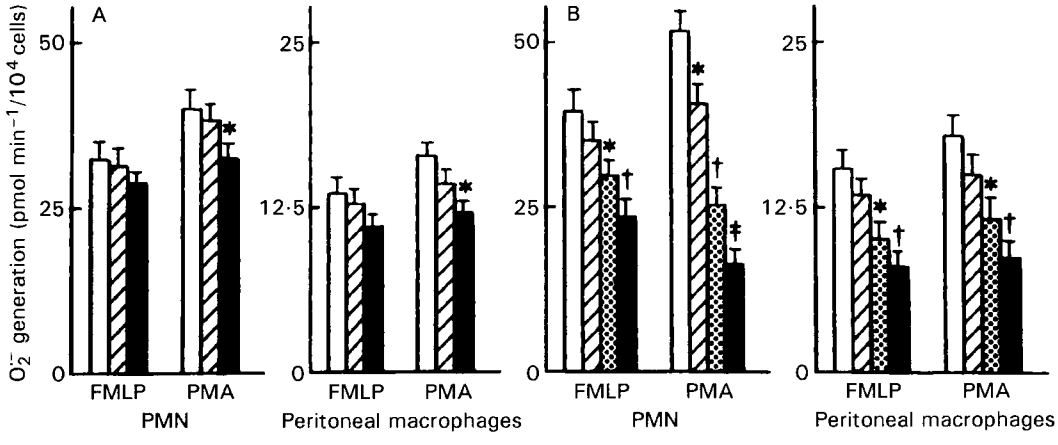

Figure 1. Influence of Twinline on in-vivo (A: □, before experiment; ▨, on 15th day; ■, on 30th day) and in-vitro (B: □, control (no Twinline); ▨, 0·01% Twinline; ▩, 0·1% Twinline; ■, 1% Twinline) O_2^- generation. Polymorphonuclear leukocytes (PMN) and peritoneal macrophages were obtained from mice fed a conventional diet (CRF-1) or Twinline for 15 or 30 days (n = 5 in each of four groups). PMN and peritoneal macrophages from Twinline-fed mice were cultured in medium without Twinline (A) whereas PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24 h in medium containing the indicated concentrations of Twinline (B). The cells were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol 12-myristate 13-acetate (PMA) and O_2^- generation was measured. *$P < 0.05$, †$P < 0.001$, ‡$P < 0.01$, significantly different from control result.

Table 2. Influence of Twinline on reactive-oxygen generation by mouse polymorphonuclear leukocytes and peritoneal macrophages in-vivo and in-vitro.

Treatment	Chemiluminescence (peak mV/5 × 10^5 cells)			
	Polymorphonuclear leukocytes		Peritoneal macrophages	
	FMLP (10^{-7}M)	PMA (50ngmL^{-1})	FMLP (10^{-7}M)	PMA (50ngmL^{-1})
In-vivo				
CRF-1 for 30 days	43·7±3·5	57·1±4·2	27·4±3·0	30·5±2·7
Twinline for 15 days	38·2±3·0*	44·7±3·8†	24·3±3·2	25·1±2·8*
Twinline for 30 days	33·7±2·9*	35·6±3·6†	20·5±2·1*	17·8±2·2†
In-vitro				
Twinline 0%	45·6±3·8	59·4±3·2	36·4±3·1	42·7±3·1
0·01%	41·8±3·2	57·1±3·0	33·5±2·7	39·1±2·9
0·1%	37·4±2·7*	53·1±2·6*	31·9±2·5	28·4±2·8*
1%	24·6±3·4†	39·5±2·8†	23·8±3·7*	22·1±2·5†

Polymorphonuclear leukocytes (PMN) and peritoneal macrophages were obtained from mice which had been fed a conventional diet (CRF-1) or Twinline for 15 or 30 days (n = 5 in each of four groups), and PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24h in medium containing the indicated concentrations of Twinline. The PMN and peritoneal macrophages were then stimulated with N-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol 12-myristate 13-acetate (PMA) and chemiluminescence was measured for 10min. *$P < 0.05$, †$P < 0.01$, significantly different from control (CRF-1) result.

NO generation, iNOS and its mRNA

NO generation from peritoneal macrophages was suppressed by Twinline both in-vivo and ex-vivo (Figure 3A). When peritoneal macrophages were treated with 0·1% and 1% Twinline for 48h NO generation was reduced to 18·0±7·0 ($P < 0.05$) and 7·9±3·8μM ($P < 0.01$), respectively, compared with the control level (27·6±8·7μM). NO generation was markedly reduced in peritoneal macrophages obtained from mice fed Twinline for 30 days; peritoneal macrophages from these mice generated 13·8±3·9μM NO whereas peritoneal macrophages from control mice generated 23·4±4·2μM NO when 1000ngmL^{-1} lipopolysaccharide (LPS) was added ($P < 0.01$). In parallel with the suppression of NO generation, iNOS, a 130kDa protein, was not observed in peritoneal macrophages obtained from Twinline-treated mice but was observed in peritoneal macrophages obtained from control (untreated) mice. The protein was weakly expressed (20% of control) after the addition of 1μgmL^{-1} LPS even by peritoneal macrophages from mice fed Twinline (Figure 4A). The level of iNOS-mRNA was also reduced by in-vitro treatment of peritoneal macrophages with 0·1% Twinline; mRNA expression was not observed in 1% Twinline-treated peritoneal macrophages (Figure 4B).

Table 3. Influence of Twinline on phagocytosis by polymorphonuclear leukocytes and peritoneal macrophages.

Treatment	Phagocytosis (%)	
	Polymorphonuclear leukocytes	Peritoneal macrophages
In-vitro		
CRF-1 for 30 days	47·3±4·6	58·4±5·1
Twinline for 15 days	45·1±4·7	56·1±4·0
Twinline for 30 days	44·7±4·5	53·9±4·2*
In-vivo		
Twinline 0%	49·0±5·2	60·4±4·0
0·01%	48·2±4·8	57·3±3·8
0·1%	47·3±4·6	56·8±4·4
1%	44·5±4·7	54·7±3·7†

Polymorphonuclear leukocytes (PMN) and peritoneal macrophages were obtained from mice which had been fed a conventional diet (CRF-1) or Twinline for 15 or 30 days (n = 5 in each of four groups), and PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24h in medium containing the indicated concentrations of Twinline. Phagocytic cells were observed microscopically. Phagocytic activity is presented as the percent of phagocytic cells/400 cells. *$P < 0.05$, †$P < 0.01$, significantly different from control (CRF-1) result.

Discussion

Reactive-oxygen intermediates have a variety of biological activity associated with the microbicidal activity of phagocytes, including tyrosine phosphorylation of proteins, inactivation of proteolytic enzymes, impairment of DNA, and induction of apoptosis (Green et al 1971; Ferrante 1989; Bhatnagar 1994; DeFranco 1994; Shoji et al 1995). This microbicidal activity is most important in the nutritional management of post-operative, traumatized, organ-transplanted, virus-infected, and advanced cancer patients. It is well known that the bactericidal and fungicidal activity of phagocytes correlates closely with the generation of reactive-oxygen intermediates (Sasada et al 1987; Ferrante 1989) and so suppression of the generation of reactive-oxygen intermediates is potentially

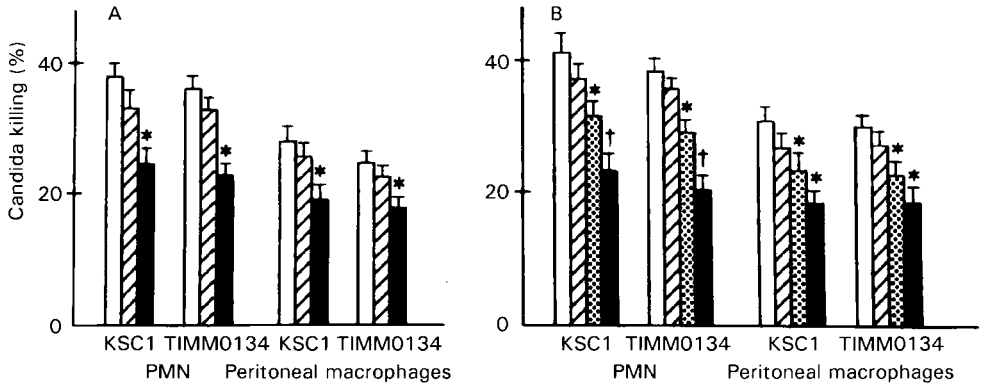

Figure 2. Influence of Twinline on the killing of candida cells (types TIMM0134 and KSC1) by polymorphonuclear leukocytes (PMN) and peritoneal macrophages obtained from mice fed CRF-1 or Twinline for 15 or 30 days (n = 5 in each of 4 groups). PMN and peritoneal macrophages from Twinline-fed mice were cultured in medium without Twinline (A: □, before experiment; ▨, on 15th day; ■, on 30th day), and PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24h in medium containing the indicated concentrations of Twinline (B: □, control (no Twinline); ▨, 0·01% Twinline; ▦, 0·1% Twinline; ■, 1% Twinline).

conducive to multiple infections. Because generation of extreme levels of reactive-oxygen intermediates and suppression of reactive-oxygen intermediate-scavenging enzymes might cause tissue damage (Halliwell 1992; Evans 1993; Laskin & Pendino 1995; Baliga et al 1997), intact regulation of the generation and scavenging of reactive-oxygen intermediates is very important for the maintenance of body integrity.

The influence of elemental diets on phagocyte function is poorly understood. Our study has shown that Twinline dose-dependently suppressed the generation of reactive-oxygen intermediates by mouse peritoneal macrophages although phagocytosis was not suppressed in-vitro. Corresponding with the suppression of $O_2^{\cdot-}$ generation and chemiluminescence, the candida-killing activity of peritoneal macrophages was reduced by Twinline. By use of other elemental diets (Nutrisourse Modular System) Shou et al (1991) also demonstrated suppression of the $O_2^{\cdot-}$ generation and the candida-killing activity of peritoneal macrophages. These results and ours suggest that elemental diets are likely to suppress the bactericidal activity of phagocytes by suppressing the generation of reactive-oxygen intermediates.

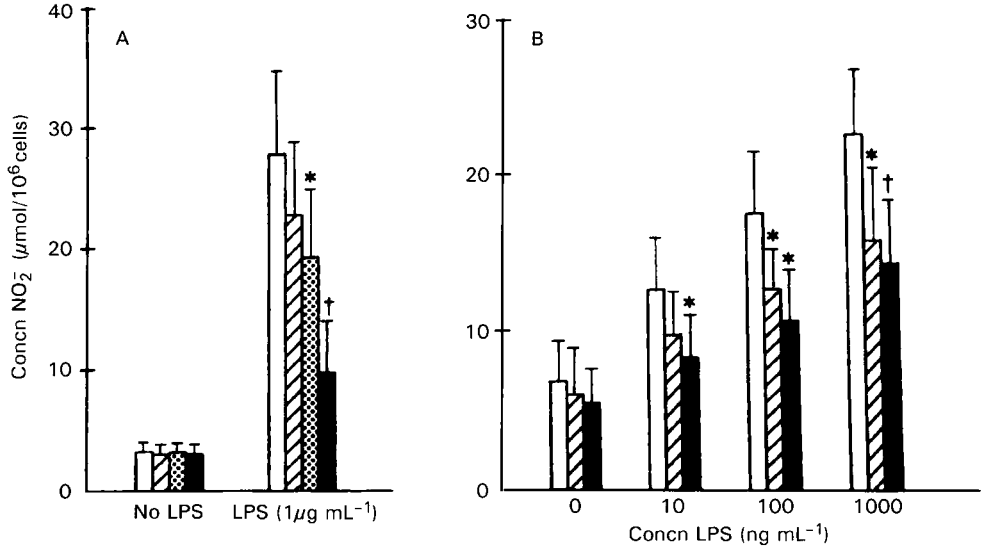

Figure 3. Ex-vivo (A: □, control (no Twinline); ▨, 0·01% Twinline; ▦, 0·1% Twinline; ■, 1% Twinline) and in-vivo (B: □, before experiment; ▨, on 15th day; ■, on 30th day) influence of Twinline on NO generation by mouse peritoneal macrophages. A: Peritoneal macrophages obtained from untreated mice (n = 4 in each group) were cultured for 48h in medium containing the indicated concentrations of Twinline and lipopolysaccharide (LPS). B: CBA/J mice (n = 4 in each group) were fed CRF-1 (control) or Twinline for 15 or 30 days, and peritoneal macrophages were collected and cultured for 48h in medium containing the indicated concentrations of lipopolysaccharide. Each bar indicates the mean ± s.d. of results from triplicate experiments. *$P < 0.05$, †$P < 0.01$, significantly different from control result.

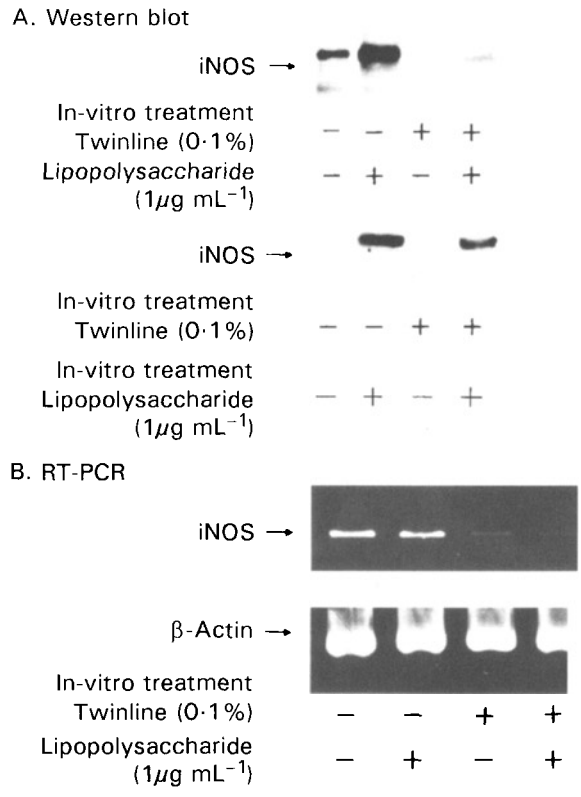

Figure 4. Influence of Twinline on inducible NO synthase (iNOS) and its mRNA expression in mouse peritoneal macrophages. Peritoneal macrophages obtained from Twinline-treated or -untreated CBA/J mice were cultured for 24h in medium containing the indicated reagents, and Western blotting (A) and 30 cycles of RT-PCR (B) were performed.

It has been reported that the serum level of peroxidized fatty acids in patients receiving Twinline was lower than that in patients receiving a conventional elemental diet containing long-chain triglycerides (Ueta et al 1997a). This difference seems to depend on the fatty-acid composition of Twinline and other elemental diets. In addition to active suppression of the generation of reactive-oxygen intermediates, the low level of peroxidized fatty acids seems to be advantageous for nutritional treatment of patients with ischaemic heart and brain disorders, because reactive-oxygen intermediates and peroxidized fatty acids are harmful in such disorders (Abadie et al 1993; Koudelova et al 1994).

Only slight suppression of $O_2^{\cdot-}$ generation was observed for peritoneal macrophages from mice fed Twinline for 15 days whereas $O_2^{\cdot-}$ generation and chemiluminescence were markedly suppressed for those from mice fed for 30 days. In addition to suppression of the generation of reactive-oxygen intermediates, human lymphocyte activity such as blastogenesis and generation of cytokines such as granulocyte macrophage-colony stimulating factor, tumour necrosis factor, and interleukin-2 were suppressed by in-vitro Twinline (data not shown). We have previously reported suppression of $O_2^{\cdot-}$ generation by PMN in Twinline-treated patients (Ueta et al 1997b). That finding and our current results suggest that Twinline has leukocyte-function-suppressing activity and so precautions against infection, especially fungal infection such as candidiasis, are essential during long-term administration of Twinline.

In conjunction with the suppression of the generation of reactive-oxygen intermediates, NO generation was also suppressed by Twinline. Suppressed NO generation was paralleled by reduced levels of iNOS protein and its mRNA. NO is synthesized from L-arginine with nicotinamide adenine dinucleotide phosphate and NOS as catalysts (Moncada et al 1991). Twinline contains less L-arginine than conventional elemental diets and it is, therefore, possible that the mechanism of the reduction of NO generation in peritoneal macrophages from Twinline-fed mice might depend on the small amount of L-arginine in the diet. It was recently reported that elemental diets containing large amounts of L-arginine, RNA and omega-3 fatty acid upregulate cellular proliferation, interleukin-2 production, and the cytotoxicity of T cells and macrophages (Daly et al 1992; VanMeter et al 1994; Kemen et al 1995; Senkal et al 1995). These reports suggest an advantageous elemental diet composition for the preservation of leukocyte function. Because Twinline downregulates leukocyte activity, including generation of cytokines, which upregulate NO generation (Liew 1995), there is a possibility that suppression of NO generation resulted, at least partially, from suppressed cytokine generation.

For nutritional management of patients, especially those in an immune-compromised condition, it is essential to recognize the physiological activity of their elemental diet. The results of this study reveal some of the characteristic effects of Twinline which might be important in its clinical use.

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Delayed Healing of Chronic Gastric Ulcer after *Helicobacter pylori* Infection in Mice

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Abstract

It has been suggested that there is a close relationship between *Helicobacter pylori* and the onset or recurrence of gastroduodenal disease. The aim of this study was to examine the effect of *H. pylori* on the healing of chronic gastric ulcers induced in mice.

H. pylori administered to nude mice delayed the healing of experimental acetic acid-induced gastric ulcers. Histological examination showed the occurrence of high densities of *H. pylori* on the surface of epithelial cells and in the ulcerated area. Repeated administration of amoxicillin (10 mg kg^{-1} daily for 5 days) eradicated *H. pylori* and increased the rate of healing of gastric ulcers in *H. pylori*-infected mice, but metronidazole, which also eradicated the organisms, did not significantly affect the rate of healing.

In conclusion, *H. pylori*-infection delayed the healing of gastric ulcers induced by the serosal application of acetic acid in mice, possibly by aggravation or prolongation of the mucosal inflammation. Amoxicillin eradicated *H. pylori* and promoted gastric ulcer healing in mice infected with *H. pylori*.

Helicobacter pylori, a Gram-negative spiral bacterium, was first isolated from a patient with chronic gastritis by Warren & Marshall in 1983. Since then, much evidence has indicated the close relationship between gastroduodenal disease and *H. pylori* (Graham et al 1987; Marshall & Langton 1986). Eradication of *H. pylori* by combined treatment with antibiotics and antacids is followed by a reduced rate of recurrence of peptic ulcers and slow resolution of the underlying gastritis (Marshall et al 1988; Perterson 1991; Graham et al 1992). It is therefore assumed that *H. pylori* might be an important pathogenic factor in ulcer recurrence. However, to clarify the clinical significance of *H. pylori* in ulcer healing the healing of gastric ulcer should be deleteriously influenced by continuous infection.

Several species such as mice (Karita et al 1994), pigs (Krakowka et al 1987; Engstrand et al 1990) and monkeys (Baskerville & Newell 1988; Shuto et al 1993; Fujioka et al 1994) have recently been used as *H. pylori* infection models. Karita et al (1993, 1994) established chronic infection of *H. pylori* in athymic mice in which *H. pylori* continuously colonized in the stomach. Other studies (Ross et al 1992; Karita et al 1994) have focused on the development of experimental acute and chronic gastritis with *H. pylori*. Despite this work it remains unclear whether *H. pylori* hinders ulcer healing in experimental animals.

This study was performed to study the healing of gastric ulcers induced in nude mice, and the effect of antibacterial drugs in *H. pylori*-infected animals.

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Materials and Methods

Chemicals

Acetic acid was from Iwai Kagaku (Tokyo, Japan), amoxicillin from Kyowa Hakko Kogyo (Tokyo, Japan), metronidazole, vancomycin and nalidixic acid from Sigma (St Louis, MO), polymyxin B from Pfizer Pharmaceutical (Tokyo, Japan), trimethoprim from Shionogi Pharmaceutical (Osaka, Japan) and 3 mg mL^{-1} amphotericin B from Bristol-Myers Squibb (Tokyo, Japan).

Animals

BALB/C athymic nude mice, 6 weeks, were obtained from Nippon SLC (Shizuoka, Japan); for

at least 1 week before use they were maintained on chow sterilized by radiation (Funabashi, Chiba, Japan). Sterile water was freely available.

Induction of gastric ulcer

Gastric ulcers were induced by serosal application of acetic acid, according to the method of Takagi et al (1969). Mice were anaesthetized with sodium pentobarbital (30 mg kg^{-1}, i.p.). The abdomen was opened and the stomach exteriorized. A metal cup (3 mm diam.) was placed at the anterior border between the corpus and antrum, and acetic acid solution (60%; 0·2 mL) was poured into the cup to make contact with the surface of the stomach. After 15 s the cup was removed, the acetic acid solution was removed by absorption into filter paper, the surface of the stomach was washed with sterile saline and the abdomen was closed by suture. Thereafter, the animals were maintained as described above.

Bacteria

A clinical isolate (CPY 2052) was kindly supplied by Dr Mikio Karita (School of Medicine, Yamaguchi University, Yamaguchi, Japan). The strain was identified by morphology, Gram's stain, urease, oxidase, catalase production, resistance to nalidixic acid and sensitivity to cephalothin, and as Cag A^+ (cytotoxin-associated gene A) and tox^+ (vacuolating cytotoxin) strain (Matsumoto et al 1997). Stock cultures were stored at −80°C in brucella broth (Baltimore Biological Laboratory, Cockeysville, MD) supplemented with 2% heat-inactivated foetal bovine serum.

Infection of mice with H. pylori

Inoculation was performed according to the methods of Karita et al (1991) and Koga et al (1996). Briefly, *H. pylori* was cultured in brucella broth supplemented with 2% foetal bovine serum. The culture was harvested, and the cells were suspended in brucella broth to 2×10^8 colony-forming units mL^{-1}. Mice were inoculated by gavage with bacterial suspension (1·5 mL) 2 days after production of the chronic ulcer. Control (non-infected) animals received vehicle alone.

Measurement of ulcerated area

Animals were killed by inhalation of carbon dioxide 6, 9, 16, 30 or 58 days after gastric ulcer production. The stomachs were removed, inflated with formalin (1%; 1 mL) and immersed in formalin (1%) for 10 min to fix the gastric wall. Subsequently, the stomachs were cut along the greater curvature and the ulcerated area (mm^2) was determined by means of a Luzex F (Nireko, Tokyo, Japan) computer-assisted image analyser system.

In other experiments *H. pylori*-infected mice with gastric ulcer were killed and the number of viable *H. pylori* in the stomach was measured. Whole stomachs were excised using sterile instruments, and the *H. pylori* colonies were counted by procedures previously reported (Koga et al 1996). Briefly, the whole stomachs were homogenized with brucella broth (2 mL) supplemented with 2% heat-inactivated foetal bovine serum, and modified Skirrow's agar plates were inoculated with diluted samples of the homogenate. Plates were incubated at 37°C in a GasPak jar for 5 days in a microaerobic environment and the colonies of *H. pylori* were then counted.

Microscopic examination

Sixteen days after ulcer production the tissue specimens were fixed in Camoy's fixing solution for 2 h, dehydrated with absolute alcohol, processed using standard procedures, embedded in paraffin, and sectioned at 4 μm. Sections were stained by the method of Genta et al (1994) which enables simultaneous visualization of *H. pylori* and gastric morphology. The sections were also stained immunologically with a monoclonal antibody specific to *H. pylori* (Institute of Immunology, Tokyo, Japan).

Effect of metronidazole and amoxicillin on ulcer healing

Either amoxicillin or metronidazole suspended in 0·4% gum tragacanth solution (10 mL kg^{-1}) was given by gavage to mice for 5 consecutive days starting 4 days after the induction of gastric ulcer. *H. pylori* was administered 2 days after ulcer production. The doses of amoxicillin and metronidazole chosen to eradicate *H. pylori* were 10 and 62·4 mg kg^{-1} daily, respectively, both administered orally. Eighteen hours after the final administration of the antibacterial drugs, the mice were killed and the stomachs were examined for ulcer area as described above.

Statistics

Data are presented as the mean ± s.e. per group. Differences between the experimental groups were determined by use of the two-tailed Dunnett's multiple comparison test; and values of $P < 0.05$ were regarded as indicative of significance.

Results

Healing of gastric ulcer

Acetic acid induced the formation of a round solitary mucosal ulcer between the gastric antrum and corpus of each mouse (Figure 1). In non-infected mice these ulcers healed spontaneously, healing being almost complete 58 days after ulcer production (Table 1). Infection with *H. pylori* delayed the healing of these ulcers; the mean sizes of the ulcers 9 and 16 days after ulcer induction were $7{\cdot}44 \pm 1{\cdot}92$ and $3{\cdot}67 \pm 0{\cdot}61\ mm^2$, respectively, 258% and 267% greater than those observed in control mice ($P = 0{\cdot}017$ and $0{\cdot}000018$).

Histopathology

The histology of a typical acetic acid-induced gastric ulcer in *H. pylori*-infected mice 16 days after ulcer induction is shown in Figure 2B. This ulcer was wide open with no re-epithelization at the edge of the ulcer. The ulcer in the non-infected mouse was small, indicating that some healing had occurred (Figure 2A). Multiple staining of the gastric mucosa clearly showed the presence of *H. pylori*; curved, rod-shaped bacteria measuring 4–6 μm were observed by light microscopy (Figures 3A and 4A). Immunohistochemical staining using a monoclonal antibody confirmed the organism as *H. pylori* (Figures 3B and 4B). High densities of *H. pylori* were present in the mucus on the surface of superficial epithelial cells (Figures 3A, B) and also in debris on the base of the ulcer (Figures 4A, B), and some were also found in both healthy and damaged mucosa. Infiltration of inflammatory cells was seen near *H. pylori* colonization in the base of the ulcer (Figures 4A, B).

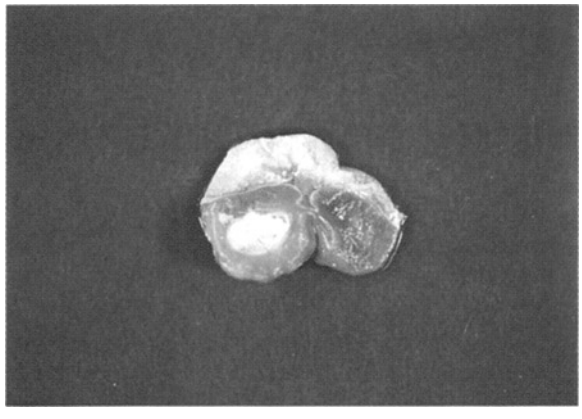

Figure 1. Gastric ulcer in mouse 9 days after induction with acetic acid.

Table 1. Healing of gastric ulcer in *H. pylori*-infected and non-infected mice.

Days after induction of ulcer	Ulcerated area (mm^2)†	
	Non-infected	Infected
6	$13{\cdot}15 \pm 2{\cdot}52$ (12)	$15{\cdot}53 \pm 2{\cdot}40$ (12)
9	$2{\cdot}08 \pm 0{\cdot}50$ (12)	$7{\cdot}44 \pm 1{\cdot}92$ (13)*
16	$1{\cdot}00 \pm 0{\cdot}24$ (10)	$3{\cdot}67 \pm 0{\cdot}61$ (17)**
30	$0{\cdot}01 \pm 0{\cdot}01$ (4)	$1{\cdot}36 \pm 1{\cdot}01$ (5)
58	$0{\cdot}19 \pm 0{\cdot}17$ (4)	$0{\cdot}56 \pm 0{\cdot}49$ (5)

†Values are means ± s.e. (the number of mice is given in parentheses). *$P < 0{\cdot}02$, **$P < 0{\cdot}001$, significantly different from result from non-infected group.

Effect of amoxicillin or metronidazole on the healing of gastric ulcers

Eradication of *H. pylori* with amoxicillin promoted the healing of gastric ulcers in *H. pylori*-infected mice (Table 2). The mean ulcer size was $4{\cdot}07 \pm 0{\cdot}61\ mm^2$ in amoxicillin-treated mice and $13{\cdot}69 \pm 1{\cdot}67\ mm^2$ in control mice ($P = 0{\cdot}000093$). No *H. pylori* were detected in the stomachs of treated mice ($< 10^2$ colony-forming units/stomach, $n = 5$). Metronidazole similarly eradicated *H. pylori* infection ($< 10^2$ colony-forming units/stomach, $n = 5$), but the healing of gastric ulcer was similar to that in the control group. The mean ulcer size was $14{\cdot}27 \pm 3{\cdot}26\ mm^2$ in metronidazole-treated mice and $13{\cdot}69 \pm 1{\cdot}67\ mm^2$ in control mice ($P = 0{\cdot}87$).

Discussion

Infection of germ-free athymic mice with *H. pylori* was developed by Karita et al (1991, 1994) who found that the bacteria persist in the stomach for more than 20 weeks. Development of gastritis and duodenitis in this mouse model after *H. pylori* colonization might be suitable for studying the clinical significance of *H. pylori*-infection (Karita et al 1991; Marchetti et al 1995). Although combination of antibacterial drugs and an anti-ulcer agent cured the *H. pylori* infection in this animal model (Karita et al 1993), the relationship between

Table 2. Effect of antibacterial drugs on healing of gastric ulcer in *H. pylori*-infected mice.

Treatment	Ulcerated area (mm^2)†
Control (*H. pylori*-infected)	$13{\cdot}69 \pm 1{\cdot}67$ (12)
Non-infected	$9{\cdot}22 \pm 1{\cdot}35$ (10)*
Metronidazole (*H. pylori*-infected)	$14{\cdot}27 \pm 3{\cdot}26$ (13)
Amoxicillin (*H. pylori*-infected)	$4{\cdot}07 \pm 0{\cdot}61$ (12)**

†Values are means ± s.e. (the number of mice is given in parentheses). *$P < 0{\cdot}02$, **$P < 0{\cdot}001$, significantly different from result for control group.

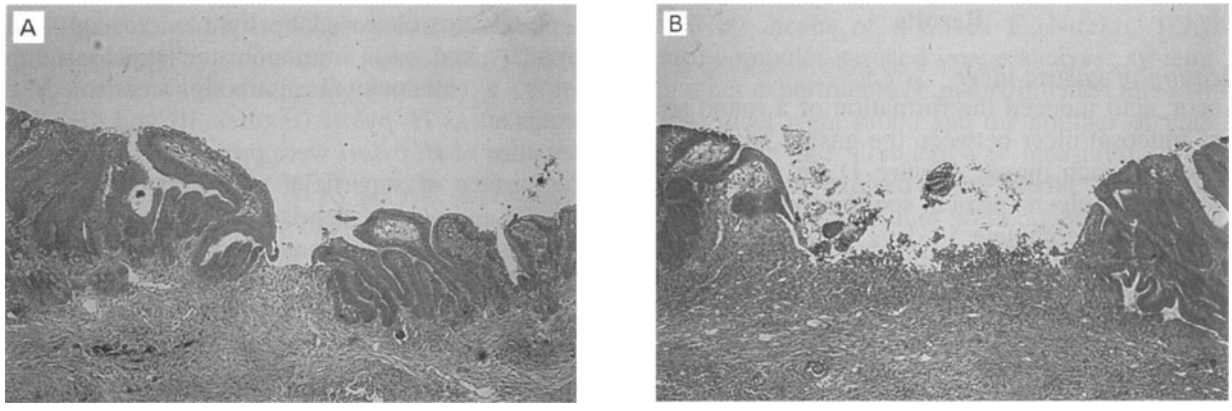

Figure 2. Multiple staining of the ulcerated area 16 days after induction in (A) non-infected mouse stomach and (B) *H. pylori*-infected mouse stomach (magnification × 74).

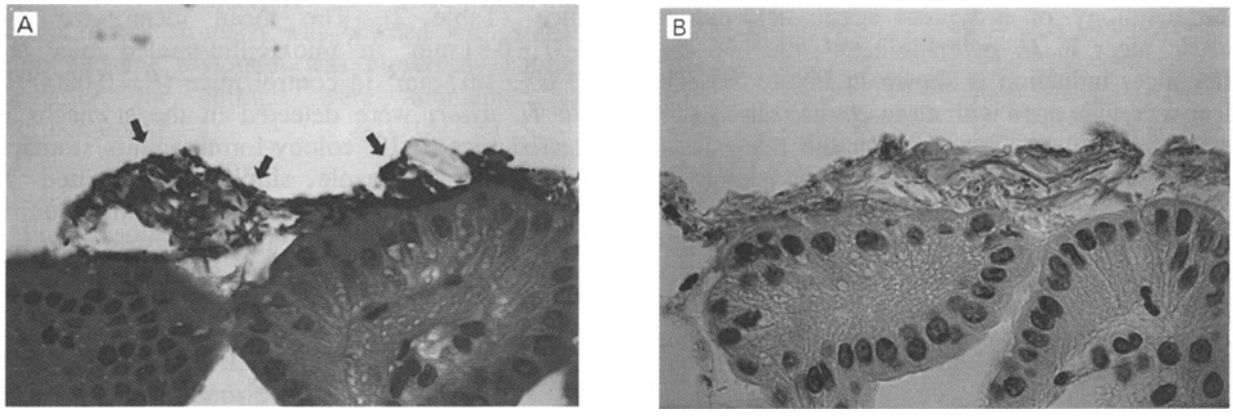

Figure 3. A. Multiple staining of the surface of the gastric mucosa 16 days after ulcer induction in an *H. pylori*-infected mouse stomach (*H. pylori* are indicated by arrows) and B. immunohistochemical identification of *H. pylori* on the surface of the gastric mucosa (magnification × 730).

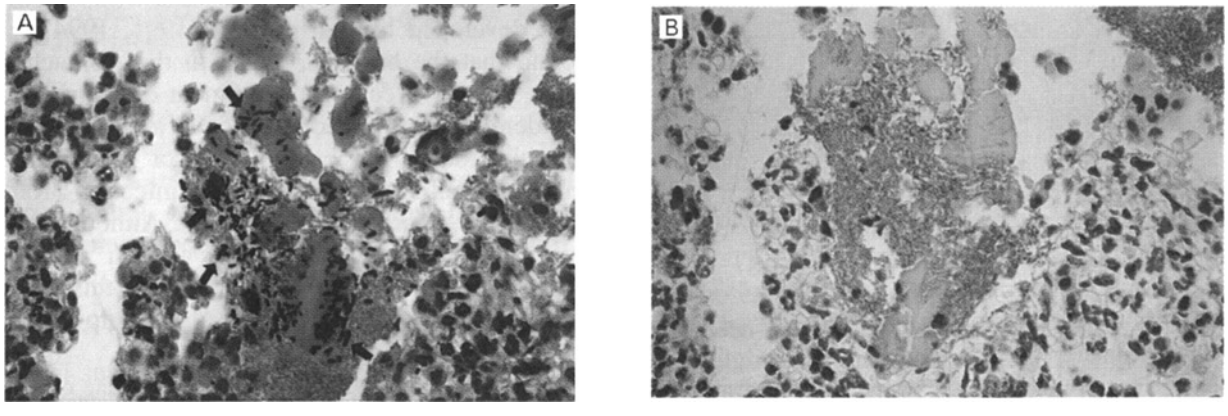

Figure 4. A. Multiple staining of debris at the base of a gastric ulcer 16 days after ulcer induction in an *H. pylori*-infected mouse stomach (*H. pylori* are indicated by arrows) and B. immunohistochemical identification of *H. pylori* in the debris at the base of the ulcer (magnification × 730).

H. pylori infection and the healing of chronic gastric ulcer has not been clearly demonstrated in the animal models. In the current study we have shown for the first time that gastric ulcer healing was significantly delayed by the continuous presence of *H. pylori*. Ross et al (1992) showed that daily gastric administration of *H. pylori* or bacterium-free filtrates caused prolongation of pre-existing gastric ulceration in rats and that *H. pylori* alone had no effect on the normal gastric mucosa of rats, although infection with *H. pylori* was not successful in their study.

H. pylori can induce the production of cytokines (Noach et al 1994; Crowe et al 1995) which are causal factors in mucosal inflammation. This suggests that *H. pylori* might aggravate or prolong the inflammation of gastric mucosa and delay ulcer healing, a contention consistent with the infiltration of inflammatory cells around the organisms in the base of the ulcer. Alternatively, the growth or integrity of gastric epithelial cells might be impaired by *H. pylori* infection. Monochloramine (Dekigai et al 1995; Murakami et al 1995) and cytotoxins (Cover et al 1991; Ghiara et al 1995) from *H. pylori* can damage gastric mucosal cells and might delay gastric ulcer healing.

H. pylori can survive persistently in the athymic mouse stomach. The organisms were clearly present in wide areas of the gastric mucosa, mostly in the gastric mucous layer on the surface epithelial cells and in the ulcer bed. These results accord with the observation of *H. pylori* in the surface mucous layer in man (Shimizu et al 1996).

Clinical studies (Marshall et al 1988; Hentschel et al 1990) indicate that eradication of *H. pylori* can be achieved by use of antibacterial drugs and an anti-ulcer agent, and that this reduces the recurrence of gastric and duodenal ulcers. Amoxicillin and metronidazole can cure the infection in *H. pylori*-infected mice (Karita et al 1993). In the current study we found that amoxicillin counteracted the deleterious influence of *H. pylori* on gastric ulcer healing. These results suggest that *H. pylori* might be a deleterious factor in gastric ulcer healing and that eradication of this organism might accelerate the healing of gastric ulcers. Because amoxicillin can protect the gastric mucosa in rats (Lam et al 1994), this action and its antibiotic effect might also contribute to ulcer healing. It is possible that metronidazole might have a noxious effect on ulcer healing irrespective of its antibacterial effect. Further studies are needed to clarify the different effects of amoxicillin and metronidazole on ulcer healing in this mouse model.

In conclusion, this study has clearly demonstrated that *H. pylori* infection delayed the healing of gastric ulcers induced by the serosal application of acetic acid in mice, possibly by aggravation or prolongation of mucosal inflammation. Amoxicillin, which eradicated the *H. pylori*, promoted gastric ulcer healing in mice previously infected with *H. pylori*.

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A Comparative Study of the Effects of Sparteine, Lupanine and Lupin Extract on the Central Nervous System of the Mouse

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Abstract

Lupin is toxic because of its alkaloid content, sparteine and lupanine in particular. Although the pharmacological properties of sparteine are well known those of lupanine have not been much studied. This paper reports procedures for extraction, purification and crystallization of lupanine, and methods for the preparation of an extract for injection of *Lupinus mutabilis* Sweet, and for the determination of the acute toxicity and maximum non-lethal dose (DL0) of lupanine, sparteine and lupin extract in the mouse.

The three substances were tested on the central nervous system (CNS) for locomotor activity, for interaction with specific drugs used for treatment of the CNS (the stimulant drugs amphetamine and pentetrazol and the depressant drugs pentobarbital and chlorpromazine) and for analgesic activity.

The results indicate that lupanine and lupin extract are less toxic than sparteine and that at the doses studied the three products have a weak sedative effect on the CNS.

Lupin, an interesting source of alimentary proteins, could be suitable for animal feed except that this member of the *Fabaceae* is toxic because of its alkaloid content, sparteine and lupanine in particular. Although the pharmacological and toxicological properties of sparteine are well known (Duke 1987; Bruneton 1993) those of lupanine have not been much studied because the compound is not commercially available. Sparteine was first extracted from *Cytisus scoparius* (L.) Link., but also occurs widely in the genus Lupin (Kinghorn & Balandrin 1984).

Sparteine has numerous pharmacological properties: cardiovascular, antihypertensive (Faucon & Ollagnier 1978; Schmitt 1980; Cohen 1981; Carraz & Carraz 1985; Piéri & Kirkiacharian 1986); effects on the autonomic nervous system including ganglion blocking and antimuscarinic effects (Lechat 1978; Schmitt 1980; Cohen 1981; Piéri & Kirkiacharian 1986); and effects on the central nervous system (CNS) including depressant (Schmitt 1980; Piéri & Kirkiacharian 1986), oxytocic (Lechat 1978; Cohen 1981; Piéri et al 1986), diuretic (Schmitt 1980; Cohen 1981) and local anaesthetic (Schmitt 1980; Cohen 1981) effects.

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Pharmacology books and other publications contain little information about lupanine. It is less toxic than sparteine; at high dose it stops the heart in diastole and at low dose it reduces coronary flow, contraction amplitude and heart rate (Duke 1987; Bruneton 1993). It is spasmolytic, oxytocic and ganglion blocking (Schmitt 1980). In the mouse, non-toxic intraperitoneal doses (LD0) of these two compounds are $30{\cdot}7\,mg\,kg^{-1}$ and $150\,mg\,kg^{-1}$, respectively for sparteine and lupanine; intraperitoneal LD100 values (i.e. the doses killing all the animals tested) are $150\,mg\,kg^{-1}$ (sparteine) and $225\,mg\,kg^{-1}$ (lupanine) (Yovo 1982). LD0 values established by other authors are $30\,mg\,kg^{-1}$ intravenous and $100\,mg\,kg^{-1}$ subcutaneous in the rabbit (Flury & Zernik 1928), $120\,mg\,kg^{-1}$ subcutaneous (Zipf & Triller 1943) and $75\,mg\,kg^{-1}$ subcutaneous in the mouse (Gordon & Henderson 1951). The LD50 (i.e. the doses killing half the animals tested) of sparteine and lupanine in the mouse are, respectively, 36 and $175\,mg\,kg^{-1}$ for intraperitoneal administration and 220 and $410\,mg\,kg^{-1}$ for oral administration (Yovo et al 1984). In the rat the intraperitoneal LD50 of lupanine was found to be $177\,mg\,kg^{-1}$ (Petterson et al 1987); LD50 values measured after oral administration are $1664\,mg\,kg^{-1}$ (Petterson et al 1987) and $1440\,mg\,kg^{-1}$ (Shani et al 1974). Intraperitoneal

LD50 values for lupanine are, therefore, similar for the mouse and rat but rats are more resistant after oral administration.

In this paper, we compare the effects of lupanine and extract of lupin (*L. mutabilis* Sweet) on the CNS with those of sparteine under the same experimental conditions. After determination of acute toxicity, the orientation test of Irwin (1962) as modified by Moreau (1975) and Foussard-Blanpin (1980) was used to evaluate the effects on animal behaviour in different situations. A series of pharmacological tests (Boissier & Simon 1962; Koster et al 1959) was then used to explore the action of sparteine, lupanine and lupin extract on the CNS.

Because lupanine is not commercially available we perfected an effective and simple method for isolation of lupin alkaloids and then optimized the conditions for obtaining pure lupanine, which was assayed by scanning densitometry after thin-layer chromatography.

Materials and Methods

Plant material

The seeds of *Lupinus mutabilis* Sweet were provided by the Institut National de la Recherche Agronomique (INRA) F-86600 Lusignan, France.

Preparation of products for injection

Lupin extract. Lupin seeds were ground and the powder was defatted with hexane in a Soxhlet extractor. The defatted powder was made alkaline with aqueous ammonia (15%) and extracted with dichloromethane. The crude extract was concentrated by evaporation under reduced pressure and purified by successive transfer to the aqueous phase (HCl 0·1 M) and to organic phases (dichloromethane, 15% ammonia).

The extract was prepared at a concentration of 100 mg/25 mL by addition of HCl (0·1 M) to pH 7 and adjusting with water to the correct concentration.

Extraction, purification and preparation of lupanine and sparteine. Lupanine was obtained in purified form from a lupin extract. The extract containing the alkaloids was subjected to column chromatography on silica gel 60 (70–230 mesh; Merck #7734) which was eluted with 100 : 5 (v/v) $CHCl_3$–CH_3OH. Successive fractions were monitored simultaneously with a test sample of lupanine by thin layer chromatography (TLC) on silica gel $60F_{254}$ (Merck #5715) (Dormeau 1992; Pothier 1995) with $CHCl_3$–acetone–28% NH_4OH, 25 : 24 : 1 (v/v) as mobile phase. After development alkaloids were detected by spraying the plates with Dragendorff's reagent (Munier & Macheboeuf 1949). The fractions containing lupanine (hR_F 66) were concentrated under reduced pressure and monitored by GC–MS (lupanine, m/z (%), 70 eV: 248 (37) (M^+), 247 (25), 150 (32), 149 (59), 136 (100), 98 (32), 97 (26), 94 (18), 69 (18), 55 (59); Kinghorn & Balandrin (1984)).

It is not easy to obtain crystalline lupanine hydrochloride because the dihydrochloride gives deliquescent prisms and the hydrochloride dihydrate is hygroscopic.

For sparteine we proceeded in the same manner with a standard of sparteine sulphate pentahydrate (Aldrich).

Densitometry

Densitometry was performed according to the TLC method of Pothier (1995). Lupin extract was spotted by means of a Linomat IV (Camag) and 2, 6, 10, 12 and 16 μg of lupanine in methanolic solution was spotted as a reference. After elution the plates were sprayed with Dragendorff's reagent as modified by Munier & Macheboeuf (1949), then read with a Camag densitometer 76510 TLC/HPTLC Scanner coupled with a Merck 2500 integrator.

Pharmacological experiments

Preparation of solutions for injection. The three basic products were acidified by adding HCl (0·1 M) to physiological pH.

Animals. EOPS male Swiss mice, 20 ± 2 g, were supplied by CERJ (53680-Le Genêt, France). For each test mice were allotted at random to several groups of five animals and kept under observation for 8 days.

Determination of acute toxicity. Increasing doses (50, 100, 125, 250 or 500 mg kg^{-1}) of sparteine, lupanine and lupin extract were administered intraperitoneally (0·5 mL/20 g) to groups of five mice. Animals were observed for eight days.

Effect on psychomotor activity. Psychomotor activity was evaluated using mice (three per group) housed in small Plexiglas cages under temperature-(20 ± 2°C) and noise-controlled conditions. The mice were submitted to various tests according to the technique described by Foussard-Blanpin (1980) and Picq et al (1991). Assays were performed before and after treatment (30 min, 1, 2, 3 h) to establish behaviour codification.

Exploration test. A naive mouse was placed on an automated hole board (16 holes Apelex) with

automatic counting of the animal's movements across the board (photoelectric cell). The assays were performed 30 min after administration of the three compounds (five mice per dose) and both locomotor activity and exploratory behaviour recorded every minute for 5 min.

Interaction with drugs acting on the CNS. The effects of stimulant drugs (amphetamine, pentetrazol) and depressant drugs (pentobarbital, chlorpromazine) dissolved in distilled water were compared in controls and in animals treated 30 min previously with the substances under study. Tests were performed on four groups of five mice; three groups were treated with the substances under study at the doses indicated in Tables 1 and 2 (five control animals were injected with an equal volume of normal saline solution). Animals were placed in transparent cages and symptoms were observed for a period of 1 h after injection and lethality after 24 h was recorded.

Antagonism of amphetamine-induced effects. Amphetamine ($70\,mg\,kg^{-1}$) injected intraperitoneally elicited an increase in agitation, aggressive behaviour, and sweat and saliva hypersecretion. A behaviour rating (or cotation index) from 0 to 5 was used, a score of 5 representing the maximum reaction of the control in the presence of amphetamine.

Antagonism of pentetrazol-induced convulsions and death. A modification of Hester's method (Hester et al 1971) was used. Pentetrazol ($125\,mg\,kg^{-1}$) was injected subcutaneously and survival time and lethality were noted after 24 h.

Antagonism of pentobarbital-induced hypnotic effect. Pentobarbital ($60\,mg\,kg^{-1}$) was injected intraperitoneally and the time necessary to induce sleep in the animals and the sleeping time were noted.

Antagonism of chlorpromazine-induced cataleptic effect. Chlorpromazine ($35\,mg\,kg^{-1}$) was injected intraperitoneally and the induced catalepsy was codified: 0, impossible to cross limbs; 10, possible to place together forelimbs and hindlimbs on the same side; 20, possible to cross forelimbs and hindlimbs on the same side; 25, possible to join forelimbs and hindlimbs on the same side and to cross them on the other side; 30, possible to cross forelimbs and hindlimbs on both sides simultaneously.

Analgesic effect. The effects of the substances were studied using four groups of five mice. The abdominal constriction test was performed by the method of Koster et al (1959)—sparteine, lupanine and lupin extract were injected intraperitoneally into three groups of mice 30 min before administration of 0·4% acetic acid (0·40 mL/20 g). Control animals (1 group) received normal saline solution (0·5 mL) under the same experimental conditions. Immediately after injection of the acetic acid each animal was isolated in an individual cage ($24 \times 11 \times 10\,cm^3$) and observed for 20 min. The number of abdominal constrictions and the amount of stretching were recorded.

Statistics

Results are expressed as means ± s.e.m.; statistical comparisons were made by use of Student's *t*-test with $P < 0.05$ being regarded as indicative of a significant difference.

Results

Determination of acute toxicity

The maximum non-lethal intraperitoneal doses (LD0) were $25\,mg\,kg^{-1}$ for sparteine and $64\,mg\,kg^{-1}$ for lupanine and lupin extract. Lethal intraperitoneal doses (LD100) were $100\,mg\,kg^{-1}$ for sparteine and $250\,mg\,kg^{-1}$ for lupanine and lupin extract. The clinical signs of poisoning: shaking, excitation and convulsions, were the same for the three products.

Table 1. Influence of intraperitoneal sparteine, lupanine and lupin extract on the effects of intraperitoneal amphetamine ($70\,mg\,kg^{-1}$) and subcutaneous pentetrazol ($125\,mg\,kg^{-1}$).

Product	Dose ($mg\,kg^{-1}$)	Amphetamine Lethality (%)	Pentetrazol		
			Latency period (s)	Survival period (s)	Lethality (%)
Control	–	80	1 ± 0	120 ± 4	100
Sparteine	13	100	129 ± 55*	628 ± 132**	100
Lupanine	32	80	101 ± 10*	398 ± 127*	100
Lupin extract	32	80	315 ± 162*	603 ± 198*	100

Values are means ± s.e.m. *$P < 0.05$, **$P < 0.01$, significantly different from control result.

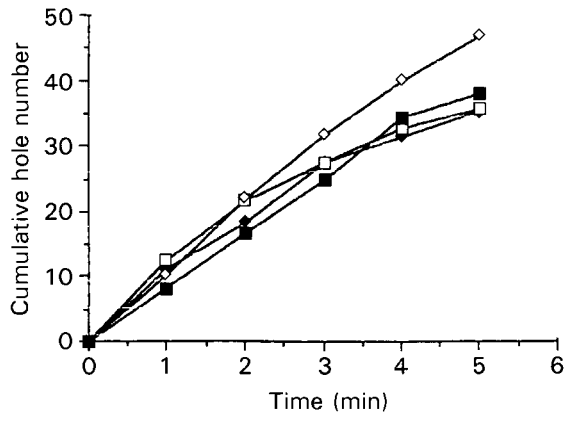

Figure 1. Cumulative number of hole pokes (exploring behaviour) in the hole-board: □, control; ◆, sparteine; ■, lupanine; ◇, lupin extract.

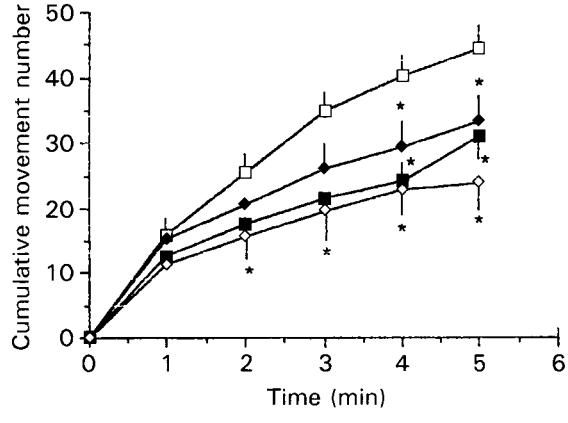

Figure 2. Cumulative number of movements (locomotor activity) in the hole-board test during 5 min observation: □, control; ◆, sparteine; ■, lupanine; ◇, lupin extract.

Effect on psychomotor activity

At the LD0 the compounds elicited a weak decrease in spontaneous activity. The tactile index and the algogenic stimulations reactivity were not modified. The behaviour indices of sedation (Boissier & Simon 1962) taking into account experimental observations were −34, −27 and −20 for sparteine, lupanine and lupin extract, respectively (Figure 1).

Exploration test

As can be seen from Figure 2, at the LD0 the three products weakly reduced locomotor activity. The number of holes explored by mice increased slightly, but not significantly, after administration of lupin extract.

Interaction with drugs acting on the CNS

Effect on stimulant drugs. The results presented in Table 1 show that the three products did not induce an antagonist effect towards amphetamine-induced stereotypy, aggression, agitation and secretion

Table 2. Influence of intraperitoneal sparteine, lupanine and lupin extract on the hypnotic effect induced by intraperitoneal pentobarbital (50 mg kg^{-1}).

Product	Dose (mg kg^{-1})	Pentobarbital	
		Sleep latency time	Sleeping time
Control	–	216±40	101±30
Sparteine	13	372±164	103±46
Lupanine	32	420±225	83±7
Lupin extract	32	246±23	85±19

Table 3. Influence of intraperitoneal sparteine, lupanine and lupin extract on the cataleptic effect of intraperitoneal chlorpromazine (40 mg kg^{-1}).

Product	Dose (mg kg^{-1})	Chlorpromazine			
		30 min	1 h	1·5 h	2 h
Control	–	10±6	12±4	16±6	27±4
Sparteine	13	11±2	16±6	19±4	15±6
Lupanine	32	19±4	19±4	18±7	23±3
Lupin extract	32	15±3	23±3	23±3	19±4

Table 4. Effect of intraperitoneal sparteine, lupanine and lupin extract on the abdominal constrictions induced by intraperitoneal administration of 0·4% acetic acid (80 mg kg^{-1}).

Product	Dose (mg kg^{-1})	Number of constrictions	Variation (%)
Control	–	30±10	–
Sparteine	13	15±6	50
Lupanine	32	12±5	60
Lupin extract	32	13±4	56

neither did they protect mice against mortality induced by pentetrazol. They did, however, delay the onset of convulsions and increase the survival time of the animals.

Effect on depressant drugs. The results presented in Table 2 show that the three substances had no effect either on the latency time or on the duration of pentobarbital-induced sleep. The cataleptic effect of chlorpromazine was not modified by sparteine, lupanine or lupin extract (Table 3).

Analgesic effect

As is apparent from Table 4 the number of abdominal constrictions and the amount of stretching induced by 0·4% acetic acid solution were slightly but not significantly reduced by the three substances.

Discussion

Several solvents have been used for extraction of lupin alkaloids. To find optimum conditions for extraction we tested two general methods: sequential extraction by maceration at room temperature and continuous extraction in a Soxhlet apparatus. Five extraction solvents were compared; from the results obtained (Table 5) it is apparent that diethyl ether–chloroform, 2 : 1 (v/v) is the most efficient both at room temperature and in a Soxhlet apparatus. Lower yields were obtained with chloroform, diethyl ether and methanol. Yields with individual solvent were not affected by whether the extraction was performed at room temperature or in a Soxhlet apparatus.

The study shows that sparteine is more toxic than lupanine. These results confirm those of Yovo (1982) and Mazur et al (1966) but not those of Couch (1926) who found lupanine to be more toxic than sparteine.

The general results obtained show that sparteine, lupanine and lupin extract have two effects on the CNS. At high doses near the LD100 (lethal dose for 100% of animals) the effects are nicotinic-like (Schmitt 1980) whereas at lower doses (< LD0) the three products have slight sedative action on the CNS, as shown by the effect on behaviour (−34, −27 and −20) (Figure 1). This last result confirms the effects reported by Piéri and Kirkiacharian (1986) for sparteine and are probably a consequence of ganglion blocking action (Schmitt 1980).

A weak depressant effect has been observed on the movement curves (Figure 2) but not on psychomotor activity. The three products modify the action of pentetrazol by increasing the latency time of convulsive attacks and by prolonging the survival time of animals (Table 1). Depressant actions were not modified (Tables 2 and 3) but the preparations were slightly but not significantly analgesic (Table 4).

In conclusion, at the doses studied sparteine, lupanine and lupin extract have a slight action on the CNS. At the lethal doses the clinical signs of intoxication are excitation (trembling and tonico-clonic convulsions). At half the LD0 the sedative effects are noticeable.

These results confirm those obtained by Nucifora & Malone (1971) who observed no specific depressive action on the CNS of rats except, at intraperitoneal doses from 55·5 mg kg^{-1}, for a reduction of motor activity in respiratory rhythm and hypothermia. Yovo (1982) used a dose equal to 0·2 × the LD50 and observed no significant effect of sparteine and lupanine on CNS activity.

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Table 5. Comparison of yield (%) of alkaloids obtained by maceration and by extraction in a Soxhlet apparatus.

Solvent	Compound	Maceration				Soxhlet			
		1	2	3	Average	1	2	3	Average
Chloroform	Sparteine	0·58	0·58	0·62	0·59	0·33	0·47	0·46	0·42
	13-Hydroxylupanine	0·37	0·40	0·39	0·39	0·31	0·39	0·34	0·35
	Lupanine	1·29	1·33	1·09	1·23	1·13	0·88	0·88	0·96
	Total	2·24	2·31	2·33	2·21	1·95	1·74	1·68	1·73
Diethyl ether	Sparteine	0·37	0·34	0·35	0·35	0·28	0·40	0·36	0·35
	13-Hydroxylupanine	0·39	0·36	0·37	0·37	0·27	0·35	0·34	0·32
	Lupanine	1·51	1·29	1·29	1·36	0·98	1·01	0·88	0·96
	Total	2·27	1·99	2·01	2·08	1·53	1·76	1·58	1·63
Diethyl ether–chloroform 2 : 1	Sparteine	0·59	0·49	0·59	0·56	0·22	0·33	0·37	0·31
	13-Hydroxylupanine	0·60	0·64	0·50	0·58	0·34	0·30	0·32	0·32
	Lupanine	1·72	1·51	1·66	1·66	1·09	1·12	1·09	1·10
	Total	2·91	2·64	2·75	2·80	1·65	1·75	1·78	1·73
Ethanol–2% acetic acid	Sparteine	0·05	0·06	0·06	0·05	0·21	0·15	0·20	0·19
	13-Hydroxylupanine	0·03	0·03	0·03	0·03	0·30	0·13	0·20	0·21
	Lupanine	0·18	0·21	0·20	0·20	1·15	0·52	0·92	0·86
	Total	0·26	0·27	0·29	0·28	1·66	0·80	1·32	1·26
Methanol	Sparteine	0·19	0·19	0·16	0·18	0·33	0·29	0·25	0·29
	13-Hydroxylupanine	0·09	0·08	0·07	0·08	0·33	0·15	0·13	0·20
	Lupanine	0·54	0·51	0·40	0·48	1·10	0·62	0·50	0·74
	Total	0·82	0·78	0·63	0·74	1·76	1·06	0·88	1·23

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