Influence of different anaesthetics on pro-inflammatory cytokine expression in rat spleen

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Summary

We examined the effect of five anaesthetic drugs commonly used in laboratory animal research (tribromoethanol, ketamine/xylazine, chloral hydrate, pentobarbital, and urethane) on the expression of four pro-inflammatory cytokines. The anaesthetic agents were applied at dosages normally used for deep surgical anaesthesia. Semiquantitative image analysis of interleukin (IL)-1β, IL-2, IL-6, and tumour necrosis factor alpha (TNFα) mRNA expression in the spleen of male Wistar rats 4 h after application of the anaesthetic drugs showed that these had moderate immunomodulatory effects. Ketamine/xylazine, chloral hydrate, and pentobarbital enhanced the basal expression of IL-1β and IL-6 mRNA in rat spleen, while urethane reduced splenic IL-1β mRNA expression. Tribromoethanol, ketamine/xylazine, and urethane reduced the basal TNFα mRNA levels, whereas TNFα mRNA expression was unaffected by chloral hydrate and by pentobarbital. The data demonstrate that these anaesthetics have slight, but significant, effects on the basal immune status of rats.

Keywords Anaesthesia; cytokines; in situ hybridization

Some anaesthetics modulate the resting immune system. Perioperative effects of such anaesthetic drugs may include a disturbed balance of pro- and anti-inflammatory cytokines in certain states of disease [McBride et al. 1996, Hunter 1999]. The perioperative-induced immunomodulatory effect of anaesthetic drugs has been suggested as the reason for the observed increase of post-anaesthetic infections [MacFarlane et al. 2000] and for the acceleration of metastasis in humans [Frid et al. 1984]. The promoting effect of anaesthetic drugs on tumour growth [Katzav et al. 1986] and/or on infections [Rubins & Charboneau 2000] has been confirmed by animal studies. Furthermore, some of the anaesthetic drugs most frequently used in small laboratory animals may induce moderate to severe tissue damage [Smiler et al. 1990].

Pentobarbital is known to cause severe tissue reactions due to its high pH value [Lumb & Jones 1984]. Urethane, a mutagenic and carcinogenic anaesthetic

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agent that is used mainly for non-survival experiments, can cause an osmotic toxicity to the mesenteric vasculature (Severs et al. 1981) or generate fluid leakage into the peritoneum (Field & Lang 1988) which could lead to ‘chemical peritonitis’ (Van Der Meer et al. 1975). Ketamine/xylazine, a widely accepted combination of drugs used for surgical anaesthesia in laboratory animals (Van Pelt 1977), caused severe tissue reactions such as muscle necrosis in rats when given intramuscularly (Smiler et al. 1990). Necrosis of abdominal wall muscle, necrosis and serositis in abdominal organs such as the spleen, and fatal peritonitis (Zeller et al. 1998) with a high rate of mortality (Greene 1997) have been described when tribromoethanol [Avertin®] has been given to rodents. When chloral hydrate has been applied, an irritancy of abdominal organs, depending on the concentration, has been described (Vachon et al. 2000). Chloral hydrate given at a high dosage of 400 mg/kg was reported to cause inflammation of the splenic capsule (Spikes et al. 1996), gastric ulcers (Ogino et al. 1990) and severe adynamic ileus, peritonitis, and death (Fleischman et al. 1977, Davis et al. 1985).

These observations demonstrate a complex influence of most anaesthetics on the immune status, on the integrity of various organ systems and on the post-anaesthetic outcome of different diseases. All of these anaesthetic agents seem to develop late local pathological effects (>24 h), especially when used at high dosages or concentrations. However, up to now nothing has been known about the early initiating mechanisms which lead to the observed inflammatory responses. Studies about the effects of these anaesthetic agents on local and/or systemic cytokine expression are absent.

To unravel a possible anaesthetic-induced local immune response as a potential post-anaesthetic mechanism of pathogenesis, we characterized the expression of the pro-inflammatory cytokines TNFα, IL-1β, IL-6, and IL-2 within the spleen soon (4 h) after application of the anaesthetic drug.

Material and methods

Animals

Adult male HsdBrl:WH Wistar rats weighing 209–215 g were purchased from Harlan-Winkelman GmbH, Borchon, Germany. After arrival the rats were housed singly in type III Makrotron cages (Scanbur A/S, Koge, Denmark) on softwood bedding (Altromin International, Lage, Germany). The animals were kept in rooms with a standardized air conditioning 20–22°C, 50–57% humidity and a 12 h artificial day/night rhythm. The rats were fed a standard diet [Altromin 1324; Altromin International, Lage, Germany] ad libitum. They had free access to tap water via water bottles. All animal experiments were approved by the RP Giessen (Az: 17a-19c20-15[1]) according to the German Animal Protection Law. The experiments were performed to teach an ‘Anaesthesia in rats’ as a module conforming to the LASA recommendations on education and training (O’Donoghue et al. 1993).

Experimental design

Rats were anaesthetized by intraperitoneal (i.p.) injection with five different anaesthetic drugs at the following dosages: 2,2,2-tribromoethanol [formerly Avertin®, Fluka, Seelze, Germany] 400 mg per kg body weight [b/w] at a volume of 7.9 ml/kg dissolved in tertiary amyl-alcohol as recommended by Hogan et al. (1986); ketamine/xylazine [Ketavet®, Pharmacia and Upjohn, Erlangen Germany/Rompun®, Bayer GmbH, Leverkusen Germany] 100 mg ketamine and 16 mg xylazine per kg b/w in a volume of 2.7 ml/kg; urethane [Fluka, Seelze, Germany] 1500 mg per kg b/w in a volume of 7.5 ml/kg; chloral hydrate [Atarost®, Atarost, Germany] 400 mg per kg b/w in a volume of 4.0 ml/kg; and pentobarbital [Narcoren®, Merial GmbH, Hallbergmoos, Germany] 50 mg per kg b/w in a volume of 1.5 ml/kg. 2,2,2-tribromoethanol, urethane, and chloral hydrate as crystalline substances were dissolved in physiological saline and passed through a sterile filter before i.p. injection. For a sham injection, saline at 0.9%
solution, 1.7 ml/kg (b/w) was used. Normal untreated rats were used as additional controls. Precautions appropriate for minimizing exposure to carcinogens were taken when handling urethane. Chloral hydrate, pentobarbital, and urethane were used at dosages recommended by Field et al. (1993). We used a high dose of 400 mg tribromoethanol per kg b/w to provoke the development of the side effects for rodents (>350 mg/kg), such as visceral adhesions and peritonitis as described by Norris and Turner (1983). We used a similar high dosage of the mixture of ketamine/xylazine as did Van Pelt et al. (1977) in rats.

Animals were killed 4 h after application of the anaesthetic drug. In order to do so, the animals were anaesthetized by inhalation of Forene® [Isoflurane; Abbott GmbH, Wiesbaden, Germany] for less than one minute. Animals were then immediately killed by intracardially injected T61 at 0.5 ml/kg b/w [Intervet GmbH, Unterschleissheim, Germany]. The spleen was removed within one minute, immediately embedded in Tissue Tek® [Miles, Elkhart, USA] and frozen in −50°C cold methylbutane [Fluka, Seelze, Germany]. The number of animals in each group was n = 3. Total number of animals used in this study was n = 18.

In situ hybridization

In situ hybridization was performed for IL-1β, IL-2, IL-6, and TNFα on 14 μm thick serial cryostat sections of the spleen. The following rat specific cDNA fragments of IL-1β, IL-2, IL-6, and TNFα were generated by reverse transcription from rat lymphatic tissue: IL-1β cDNA is a 589 bp fragment ranging from bp 206 to bp 795 of IL-1β cRNA [Acc. number M98820]; IL-2 cDNA is a 505 bp fragment ranging from base 138 to base 542 [Acc. number M22899]; IL-6 is a 206 bp fragment ranging from bp 232 to bp 437 [Acc. number M26744]; TNFα cDNA is a 291 bp fragment ranging from bp 4432 within Exon 1 to bp 5348 within Exon 3 of the TNFα gene [Acc. number L00981]. All templates were cloned into the pGEM-T vector [Promega, Mannheim, Germany]. For in vitro transcription cDNAs were linearized with the appropriate restriction enzymes and 35S-labelled sense and antisense ribonucleotide probes were generated by in vitro transcription using SP6 or T7 polymerases (Boehringer Mannheim, Germany), as appropriate for the presence of 35S-UTP (Amersham Biosciences, Freiburg, Germany). All labelled cRNAs were purified over Micro Bio-Spin[r] Chromatography columns [Bio Rad, München, Germany] and diluted in hybridization-buffer (100 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.5 mg/ml t-RNA, 0.1 mg/ml sonicated salmon sperm DNA, 1x Denhardt’s, 10% dextrane sulfate, 50% formamide) to 50.000 cpm/μl. In situ hybridization was performed by usage of radioactive-labelled cRNAs for 16–18 h at 56°C in a moist chamber. Sections were than washed in 2x SSC and 1x SSC and single-stranded RNA was digested by 10 μg/ml RNase and 1 U/ml T1 RNase [both from Boehringer Mannheim, Mannheim, Germany] in Tris/EDTA, pH 8.0, 150 mM NaCl for one hour at 37°C. Afterwards, sections were desalted by passing them through 1x SSC, 0.5x SSC, 0.2x SSC and washed in 0.2x SSC at 60°C for one hour. Then, the tissue sections were washed in H2O, dehydrated by ethanol, and air dried. Autoradiograms were taken by exposing the sections to an X-ray film [Hyperfilm-βmax, Amersham Biosciences, Freiburg, Germany] for 1–3 days. The strength of in situ hybridization signals was quantified by quantitative densitometry measurements using the MCID image analysis system (Image Research, Ontario, Canada) as follows. An empty region of the X-ray film was scanned and a ‘Flat Field’ correction according to the MCID instruction manual was performed to correct irregularities in illumination across the field of view. Density was calibrated to radiolabelled concentration standards [ARC146, American Radiolabeled Chemical Inc., St Louis, USA] which had been exposed to the same X-ray film as the hybridized tissue slices, and a standard curve that related the system’s internal density measurement units to isotope concentrations was created. For each sample the
integrated optical density, which is the mean density value of all the pixels contained within the region of interest, was measured and expressed as median relative optical density (ROD). The optical density of the X-ray film close beside each sample was defined as physical background and subtracted from the sample. For statistical analysis the mean median ROD was calculated for each experimental group. For this, three animals per group \( n = 3 \) with four spleen tissue slices \( s = 4 \) per animal were used (absolute numbers for mean relative density with variations \( \pm 12 \) per group). To calculate statistical differences between sham-treated control and anaesthetized animals, the one-way analysis of variance (ANOVA) was used.

**Results**

Quantification analysis of the *in situ* hybridization signal seen on X-ray films shows that all anaesthetic agents influenced the expression levels of some of the pro-inflammatory cytokine-mRNAs tested (Table 1). Figure 1 shows the calculated mean levels of IL-1\( \beta \), IL-2, IL-6, and TNF\( \alpha \) mRNA given as the relative optical density.

We found that i.p. injection of ketamine/xylazine, chloral hydrate, and pentobarbital significantly enhanced the basal expression of IL-1\( \beta \) mRNA, whereas urethane slightly reduced the splenic IL-1\( \beta \) mRNA levels (Fig 1A). These changes can be seen in autoradiograms of spleen tissue slices as demonstrated in Fig 2. Tribromoethanol had no effect on the low constitutive expression of IL-1\( \beta \) mRNA (Figs 1A, 2). Sham injection of physiological saline solution did not influence the low basal expression of IL-1\( \beta \) mRNA present in the spleen of normal untreated rats (Fig 1A). None of these drugs or sham injection of physiological saline induced the T cell-specific mitogen IL-2 (Fig 1B, Table 1). To prove the successful operation of the IL-2 *in situ* hybridization experiments, spleen tissue slices from rats, which were stimulated by the T-cell specific staphylococcal superantigen A (SEA) as described in detail by Bette *et al.* (2003), were incorporated as positive controls. An abundant amount of IL-2 mRNA in the spleens of SEA-stimulated animals can be seen by autoradiography (inset in Fig 1B) and measured by semiquantitative image analysis (Fig 1B). Interleukin-6 mRNA was slightly but significantly increased by ketamine/xylazine, chloral hydrate, pentobarbital and urethane, but it was not influenced by tribromoethanol (Fig 1C, Table 1). The constitutive expression of TNF\( \alpha \) mRNA was reduced by tribromoethanol, ketamine/xylazine and urethane, but was not influenced by chloral hydrate or pentobarbital (Fig 1D, Table 1). Sham injection did not significantly alter the observed splenic IL-6 or TNF\( \alpha \) mRNA levels.

**Discussion**

The analysis of five commonly used anaesthetic agents revealed that each of these substances had significant early effects on the basal cytokine mRNA expression in rat spleen. This suggests that the characteristic local side effects generated by i.p applied

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<tr>
<th>Table 1</th>
<th>Alteration in splenic cytokine mRNA expression induced by different anaesthetic substances</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>IL-1( \beta )</td>
</tr>
<tr>
<td>Sham control</td>
<td>Basal expressed</td>
</tr>
<tr>
<td>Tribromoethanol</td>
<td>↔</td>
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<tr>
<td>Ketamine/xylazine</td>
<td>↑</td>
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<tr>
<td>Chloral hydrate</td>
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<td>Pentobarbital</td>
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<td>Urethane</td>
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anaesthetics, with dominance in the pathology of abdominal organs as described in the literature (Fleischman et al. 1977, Spikes et al. 1996, Zeller et al. 1998, Vachon et al. 2000), might be based at least in part on early local synthesis of pro-inflammatory cytokines and might have induced regional inflammatory processes.

Our study focused on the local gene expression of the pro-inflammatory cytokines IL-1β, IL-2, IL-6, and TNFα in the spleen. We used the spleen, because the spleen is a prominent marker organ in different peritonitis models and therefore can be used as an abdominal organ representative for other lymphatic organs. Interleukin-1β, IL-6, and TNFα are markers...
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for early stress response in many different situations of stress. For example, IL-1β is an accepted marker for tissue reactions and inflammation in pain-induced stress (Fu et al. 1996), IL-6 is a marker for mechanical trauma or surgical stress (Fu et al. 1996) and TNFα is a marker for bacterial endotoxin or enterotoxin-induced immune stimulation (Bette et al. 1993).

In the present study, isoflurane was used as a volatile anaesthetic at the end of the experiments directly before killing the animals with T61. In this context it must be taken into account that immune modulatory properties have been reported for isoflurane when used as a volatile anaesthetic in humans. Enhanced expression of IL-1β, IL-8, and TNFα in alveolar macrophages (Kotani et al. 1999) and reduced IL-1β and IL-2 serum in venous samples of patients (Helmy et al. 1999) when administered over a period of 2 h have been described. The very short period of time of euthanasia at the end of the experiment (less than 2 min of inhalation of isoflurane and intracardial injection of the muscle relaxants T61) makes it highly unlikely that these procedures influenced the experimental outcome.

We found that ketamine/xylazine, chloral hydrate and pentobarbital anaesthesia

Fig 2 Autoradiographic detection of IL-1β mRNA by in situ hybridization analysis in rat spleen of sham-treated animals receiving saline solution (A) or different anaesthetic drugs (B–F) 4 h after application. Bar in A: 200 μm
increased the IL-1β expression in the spleen 4 h after the injection of these drugs. It is interesting that, in a later phase of observation, ketamine-induced muscle tissue lesions (Smiler et al. 1990), pentobarbital (pH-dependent)-induced severe tissue reactions (Lumb & Jones 1984) and chloral hydrate-induced abdominal tissue lesions (Vachon et al. 2000) were found. Surprisingly, there was no increased basal expression of IL-1β and IL-6 in tribromoethanol anaesthesia, but rather a decrease of the basal TNFα expression. We also found a significant decrease of IL-1β and TNFα mRNA in urethane-induced anaesthesia, demonstrating that a single injection of urethane did not cause an early inflammatory response. In this context, Kotanidou and colleagues (1996) described urethane as having more of a ‘therapeutic capacity’, which protects rats from a lethal endotoxia and reduces the TNFα release. However, Van Der Meer et al. (1975) found a ‘chemically-induced peritonitis’ when this substance was given at a high dosage.

A deep anaesthesia with tribromoethanol induced by a high dosage of 400 mg/kg injected i.p. did not enhance the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and IL-2 but rather decreased splenic TNFα mRNA levels within the early phase (<4 h). This indicates that a single injection with this substance did not initiate ‘chemically-induced peritonitis’ by inflammation/infection in the abdomen. Weiss and Zimmermann (1999) did not find any microbial infections in mice when the powdery tribromoethanol was dissolved as recommended by Hogan et al. (1986) and sterile filtration was performed before injection. However, Zeller et al. (1998) found a serositis and necrosis in mice, in spite of sterile-filtered tribromoethanol. Our findings in rats tend to support the argument that neither tribromoethanol nor urethane are likely to induce any of the described side effects after their first injection (Hogan et al., 1986). Additionally, we found that chloral hydrate and pentobarbital increased IL-6 expression. But we do not know whether this enhanced early expression of IL-6 or the other pro-inflammatory cytokines, which we found after the anaesthesia with chloral hydrate, is the initial step for a chloral hydrate-induced abdominal tissue lesion described in the literature (Vachon et al. 2000). Finally, there was no induction of IL-2 mRNA by any of the anaesthetic agents we used. This suggests that the modulatory effect of all the anaesthetics took place on the level of the unspecific immune system, especially monocytes/macrophages. However, further research with repetitive anaesthesia on early and/or late effects in analysis of pro-inflammatory cytokine levels and of correlation to possible histopathological data might be necessary to gain a better understanding of the anaesthesia-induced side effects.

In conclusion, these data show that the effects of intra-abdominal injected anaesthetics on the immune system should always be taken into consideration. The usage of ketamine/xylazine, chloral hydrate, pentobarbital, and urethane should be avoided when studies are being designed to analyse short-term effects on the immune system.

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