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Implant exudate leucocyte response to anti-inflammatory drug treatment

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Implantation of artificial materials elicits a local inflammatory response. In this study a chamber model technique, allowing sampling of the inflammatory exudate for further analysis *in vitro*, was used. Male Sprague–Dawley rats were injected daily with two different anti-inflammatory drugs, betamethasone and indomethacin, and the local cellular response was compared with a control group. The retrieved exudate was evaluated with respect to the number of leucocytes, cell viability, differential counts and serum-opsonized zymosan stimulated chemiluminescence (CL). In all groups the majority of cells were polymorphonuclear granulocytes (PMNGs). Betamethasone and high-dose indomethacin (1.92 mg kg^{-1} body weight day^{-1}) treatment caused a marked reduction in the number of accumulated leucocytes 6 days after implantation. A substantial inhibition of the CL response was observed 6 days after treatment with betamethasone (4.23 mg kg^{-1} body weight day^{-1}). An increased CL responsiveness was observed after 24 h with low-dose indomethacin (0.03 mg kg^{-1} body weight day^{-1}) and after 6 days with high-dose indomethacin (1.92 mg kg^{-1} body weight day^{-1}) treatment. In summary, depending on the anti-inflammatory drug treatment, dose and time after implant surgery, either an inhibition or stimulation of leucocyte accumulation and activation was observed. This study shows the possibilities of sampling the inflammatory exudate adjacent to a biomaterial implanted *in vivo*. This chamber model may be useful for the analysis of the inflammatory reaction around an implanted biomaterial during pharmacological treatment.

1. Introduction

Implanted artificial materials elicit specific cellular responses during inflammation and wound healing in soft as well as in hard tissues, which ultimately lead to healing or failure of the implant [1–4]. An important part of the healing is the initial inflammatory response, which is partly dependent on the biomaterial used [3–6]. Anti-inflammatory drugs might influence the composition of the inflammatory exudate and the interaction between the implant and the surrounding soft tissue. However, the effects of anti-inflammatory drugs on cell function and healing around implants are only fragmentarily known.

Glucocorticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used clinically as anti-inflammatory drugs. The anti-inflammatory drugs influence more than one step of the leucocyte recruitment and the subsequent behaviour in the inflammatory process [7–12]. *In vivo* studies have shown that glucocorticosteroid administration is followed by an increased intravascular leucocyte pool, possibly mediated by altered steroid-induced cytokine levels [13]. *In vitro* studies have shown that glucocorticosteroids cause a diminished adhesion of human granulocytes to artificial surfaces [11, 14–16] and a decrease of PMNG chemotaxis [14, 17–20], combined with an elevated random migration of leucocytes [21].

The NSAID indomethacin has several effects on inflammatory events *in vivo*: a reduction of tissue oedema [22, 23] and inhibition of leucocyte accumulation [23, 24]. However, leucocyte chemotaxis *in vitro* is decreased only with high concentrations of indomethacin and is dependent on the cell type and chemotactic stimulus [17, 25, 26].

In this study we examined the effect of a glucocorticosteroid (betamethasone) and indomethacin on infiltration and activation of inflammatory cells in the exudate inside of a chamber placed in rat muscle. The aim of the study was to determine the influence of the anti-inflammatory drugs on the cellular composition and the cellular metabolic activation (CL) of inflammatory cells by analysis *ex vivo* of the retrieved exudate from the inside of the chamber.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing about 260 g were used (Table I). The rats were anaesthetized at implantation by an intraperitoneal injection of 0.4 ml of a mixture of apozepam (5 mg ml^{-1}; Apothekarnes Laboratorium AS, Norway), nembutal (60 mg ml^{-1}; Mebumal vet, Nord Vacc. Sweden) and saline in a 2:1:1 proportion. The rats were killed by an intraperitoneal overdose (1 ml) of the above mixture.

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TABLE I The number of rats, differential counts (% PMNG) and viability (%) in each group studied. Values are means ± SEM

	Dose (mg day^{-1})	No. of rats		Differential count (% PMN)		Viability %	
		24 h	6 days	24 h	6 days	24 h	6 days
Control		5	6	94.3 ± 1.5	90.4 ± 0.9	93.4 ± 1.7	75.5 ± 8.7
β-Methasone	0.035	6	6	92.8 ± 1.0	90.5 ± 2.2	96.5 ± 0.6	84.5 ± 2.6
	1.1	6	6	85.3 ± 1.9	92.2 ± 1.8	93.2 ± 0.6	83.7 ± 1.5
	2.2	6	6	95.2 + 0.7	92.3 + 1.5	91.2 + 4.1	54.7 + 10.7
Indomethacin	0.009	6	5	96.4 ± 0.7	93.8 ± 0.9	95.3 ± 1.0	88.1 ± 3.2
	0.5	6	6	88.0 ± 1.9	88.2 ± 1.0	96.2 ± 1.3	84.8 ± 4.0

2.2. Implants and surgical procedure

The chamber technique has been described in detail previously [4]. In brief, an incision on the right side of the midline was made and a polytetrafluoroethylene (PTFE) chamber was placed on the peritoneum. Thereafter the first dose of the anti-inflammatory drugs was injected intramuscularly in the right hindleg.

2.3. Pharmacological treatment

Two anti-inflammatory drugs were used: betamethasone (lot 2 F-0384, Sigma, St Louis, Missouri, USA) and indomethacin (lot 117F-0595, Sigma, St Louis, Missouri, USA). The substances were dissolved in 0.05 ml dimethylsulphoxide (DMSO) (Fluka AG, Switzerland) and diluted in sterile water to a volume of 0.1 ml (injection volume). Each rat was injected once daily intramuscularly in the right hindleg with one of the following drugs: betamethasone 0.035, 1.1 and 2.2 mg (i.e. 0.13, 4.23 and 8.47 mg kg^{-1} body weight) or indomethacin 0.009 and 0.5 mg (0.03 and 1.92 mg kg^{-1} body weight). The doses of the drugs used were based on clinically therapeutic doses and experimental findings (unpublished data) of 50% inhibition of the rat leucocyte metabolic cell activity using *in vitro* CL measurements. Control rats were injected intramuscularly daily with 0.1 ml of a mixture of 0.05 ml Hank's balanced salt solution (HBSS) and 0.05 ml DMSO.

2.4. Exudate retrieval

The rats were killed after 24 h or 6 days by an intraperitoneal injection of 1.0 ml of a nembutal–apozepam mixture in a 1:1 proportion. The exudate within the chamber was collected by washing the inside of the chamber with HBSS (5 × 30 µl) using an automatic pipette with an attached syringe, giving a final total volume of about 150 µl for further analysis *in vitro* [4]. Cell viability was determined by trypan blue dye exclusion. Due to the small amount of sample, the number of leucocytes was counted manually in a Bürker chamber [27]. Differential counts were made on glass-smear preparations stained with Ehrlich's haematoxylin and eosin.

2.5. Chemiluminescence

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione,

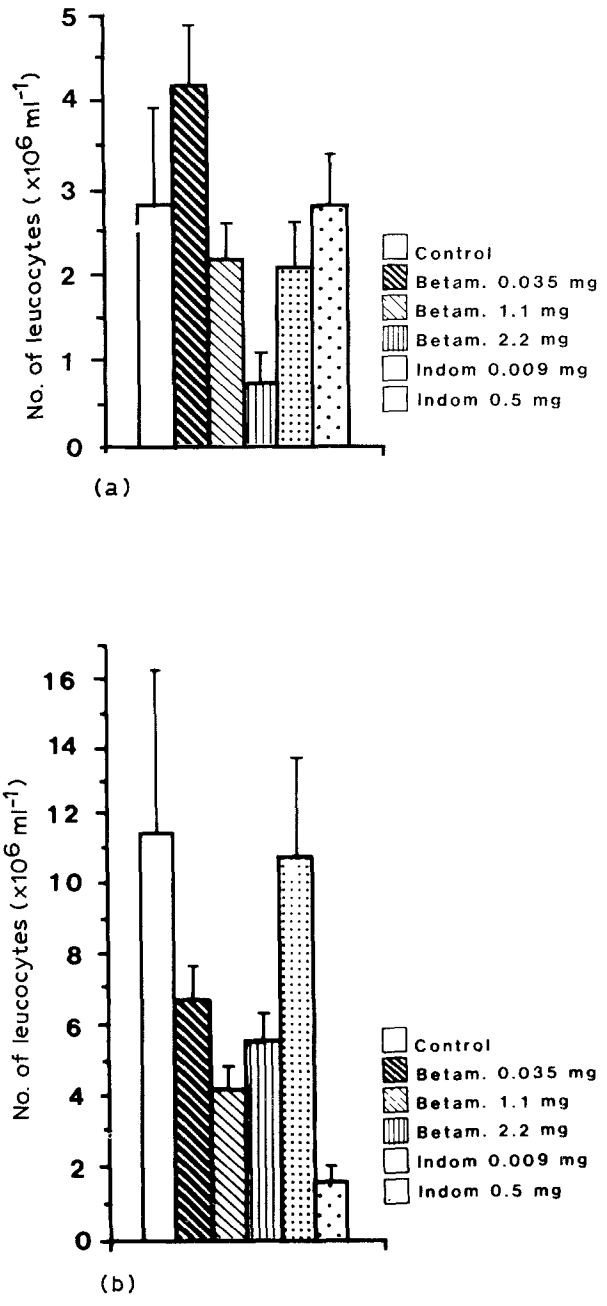

Figure 1 The numbers of leucocytes in the exudate retrieved (a) 24 h and (b) 6 days after insertion Values are means ± SEM.

Sigma, St Louis, Missouri, USA) was dissolved in DMSO at 5.5 mg ml^{-1} and diluted 1/20 in HBSS. Zymosan particles (Sigma, St Louis, Missouri, USA) were washed in HBSS and incubated with normal rat serum (12.5 mg ml^{-1}) for 30 min at 37 °C, washed

three times and resuspended in HBSS at the original concentration. 0.1 ml of the leucocytes (0.5×10^6 cells ml^{-1}) was kept on ice after retrieval from the chamber and was pre-incubated in the assay tube for 10 min at 37 °C before measurements. After the addition of luminol (0.1 ml) and serum-opsonized zymosan (0.1 ml) the CL was recorded in a luminometer (LKB 1251, LKB, Wallac, Finland) for 30 minutes.

3. Results

The number of leucocytes in the exudate (Fig. 1a and b) in the control groups increased between 24 h and 6 days (from 2.8×10^6 to 11.5×10^6 cells ml^{-1}). In the betamethasone groups (1.1 and 2.2 mg) the number of leucocytes in the exudate was reduced in a dose-dependent manner after 24 h. A decreased number of leucocytes was found in all betamethasone groups 6 days after implantation. In comparison with the control group, treatment with indomethacin had only a minor effect on the number of leucocytes in the chamber exudate 24 h after implantation. However, after 6 days indomethacin in the highest dose used (0.5 mg) caused a substantial reduction of the number of exudate cells. In both control and treated groups differential counts showed a predominance of PMNGs (Table I). The majority of cells harvested from the exudate after 24 h were viable (> 91% of the cells excluded trypan blue; Table I). The viability was diminished after 6 days treatment in all groups. In particular, the viability of leucocytes was reduced in rats treated with the highest dose of β-methasone (2.2 mg).

The metabolic activation of exudate leucocytes was evaluated by measuring the CL response in leucocytes after stimulation with serum-opsonized zymosan particles. The peak CL level in exudate-leucocytes from control rats was similar 24 h and 6 days after surgery (Fig. 2a and b). 24 h after implantation the lowest CL response was detected in leucocytes from betamethasone-treated (2.2 mg) rats. An increased CL responsiveness was detected after 24 h (but not at 6 days), with leucocytes obtained from chamber exudates in rats treated with a low dose of indomethacin (0.009 mg). After 6 days leucocytes from the high-dose indomethacin (0.5 mg) treatment group showed a markedly increased responsiveness compared with the control and low-dose indomethacin groups. A reduced CL responsiveness was observed after 6 days treatment with betamethasone (1.1 mg).

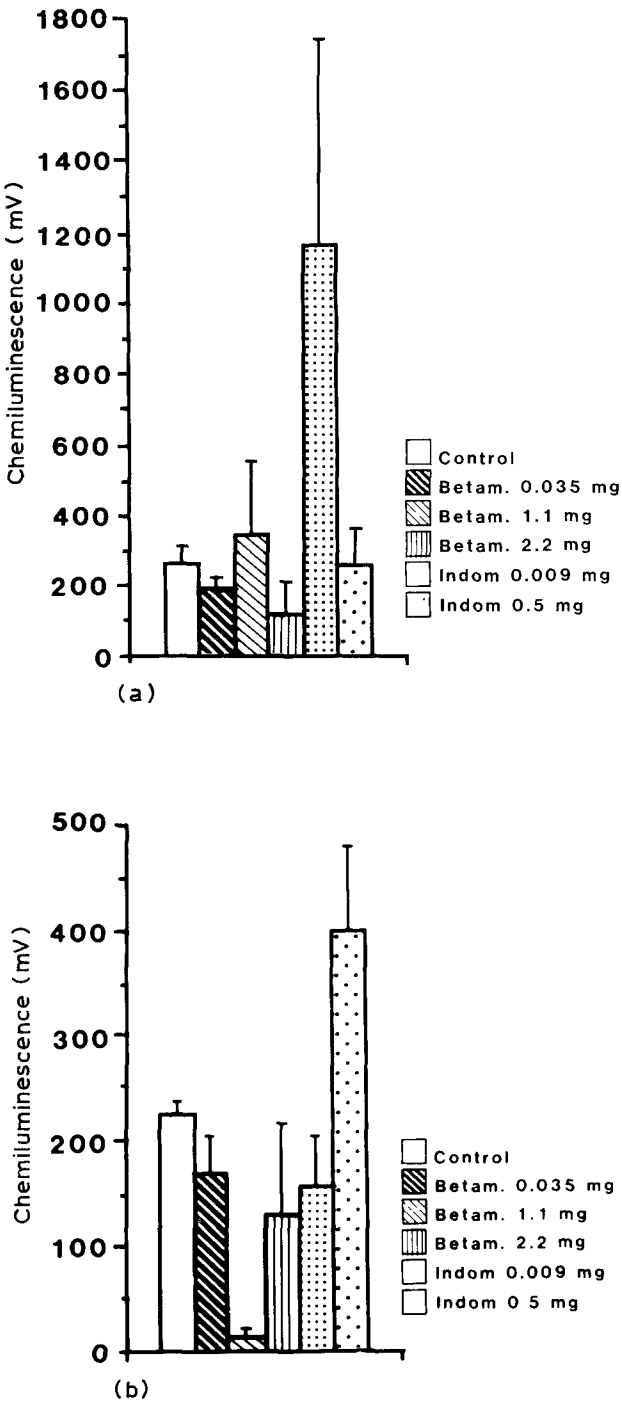

Figure 2 The exudate CL retrieved (a) 24 h and (b) 6 days after insertion. All data are given in millivolts (mV, peak levels). Values are means ± SEM.

4. Discussion

After implantation of a biomaterial, several inflammatory events are elicited, including an accumulation of leucocytes in the tissue adjacent to the material. In this study the implantation of a PTFE chamber caused an influx of leucocytes to the interior of the chamber with an increased number of leucocytes up to 6 days, corroborating previous findings using this model [4, 28]. Apart from the lowest dose of betamethasone after 24 h, the betamethasone-treated rats had a reduced accumulation of leucocytes in the chambers at both 24 h and 6 days. Our findings of an inhibition of leucocyte accumulation in the chamber exudate by betamethasone support previous observations of a reduced chemotaxis by glucocorticosteroids [14, 17–20]. Several mechanisms for the anti-inflammatory effect of glucocorticosteroids have been suggested: interaction with phospholipase A_2 and lipomodulin formation and decreased synthesis of cyclo- and lipoxygenase products [22, 29–31], effects on calcium fluxes [32, 33] and inhibition of messenger-ribonucleic acid and protein expression post-transcriptionally [17, 32–34]. However, other authors did not find any reduction of leucocyte adhesion, neutrophil LTB4 formation, random migration or chemotaxis during glucocorticoid treatment *in vitro*,

and have therefore postulated that the glucocorticosteroids block the production of mediators involved in the recruitment process [16, 25, 35].

A similar inhibitory effect on migration of leucocytes into the chambers was observed after 6 days treatment with indomethacin in a high dose. This finding is in agreement with previous *in vivo* studies (carrageenin-induced inflammation) by Higgs *et al.* [24] and *in vitro* experiments by Wildfeuer (human leucocytes) [17]: a reduction in leucocyte migration is observed only with a high dose of indomethacin ($> 1.9\ mg\ kg^{-1}$ body weight day^{-1}). The mechanism for the inhibitory effect on leucocyte migration by indomethacin is not clear. Indomethacin interacts with the cyclo-oxygenase enzyme, leading to a subsequent decrease in prostaglandin synthesis [22, 31, 36]. However, objections against inhibition of eicosanoids as a sole anti-inflammatory action of NSAIDs have been raised due to a failure to register any differences between the inhibition of cyclo- and lipoxygenase products during NSAID treatment *in vitro* [37, 38]. NSAIDs interfere with signal transducting G-protein-dependent events in neutrophils [39, 40], modulate the adenosine 3′, 5′ cyclic monophosphate level and increase the membrane viscosity [39–41]. Moreover, under *in vitro* conditions NSAIDs such as indomethacin and piroxicam suppress several PMNG functions involved in the inflammatory response, such as agonist-induced upregulation of complement receptors, aggregation, superoxide anion production and enzyme release, but the influence of NSAIDs depends on the type of stimulus and the NSAID tested [42–45]. It is possible that the inhibition of leucocyte accumulation during high-dose treatment with indomethacin may be due to an interference also with other parts of the inflammatory reaction around an implant *in vivo*. Indomethacin inhibits interleukin-1 (IL-1) production [32] and recent studies have shown that macrophages release IL-1 on polymer [46] and titanium [47] surfaces.

When examining the CL after stimulation of the exudate cells with serum-opsonized zymosan *in vitro*, the CL levels in control rats 24 h and 6 days after surgery were essentially the same. In a recent study (using titanium chambers) [48] the peak in exudate leucocyte CL responsiveness to serum-opsonized zymosan was observed 8 h after implant insertion (about 300 mV) and reached a steady-state level of about 40 mV at between 24 and 72 h. In this study the CL responsiveness was markedly higher after 24 h (about 275 mV). These experiments indicate that PMNG CL responsiveness in the exudate may be influenced by the material used.

Depending on the time after surgery and concentration of drug, the CL responsiveness of the exudate leucocytes was either stimulated or inhibited in comparison with control rats. A marked inhibition of CL was observed after 6 days treatment with betamethasone (1.1 mg), whereas a stimulatory effect was noted with indomethacin after 24 h (low concentration) and 6 days (high concentration). Previous experiments on the oxidative burst and emission of light during activation of PMNGs have suggested that oxygen-derived radicals are involved in the response [49, 50]. Furthermore, the arachidonic acid metabolism is linked to the CL reaction [51]. In this study the inhibition of leucocyte accumulation was not always accompanied by a reduction of the CL in the exudate cells when stimulated *in vitro* with the zymosan particles. After 6 days betamethasone (1.1 mg) treatment the exudate leucocytes had a markedly reduced CL responsiveness. In contrast, despite a marked reduction of leucocyte accumulation after 6 days in rats treated with the highest dose of indomethacin (0.5 mg), the exudate leucocytes from these rats had an increased CL responsiveness. The reason for the varying effects on separate functions of the rat PMNG in this study is not clear. However, previous observations *in vivo* have suggested that the migratory response and the subsequent phagocytosis process with degranulation and metabolic activation are separate events (immune complex-induced synovitis in rabbits) [52] and possibly differently regulated [53]. Moreover, NDGA, but not indomethacin, has been shown to inhibit immune complex and zymosan-induced CL in both blood and exudate PMNGs [52]. One explanation for the markedly inhibitory effect on the migratory response and the concomitant increased CL responsiveness after treatment with indomethacin might be that the inflammatory cells at different locations (vascular versus chamber exudate) constitute two pools of cells that respond to stimuli and drugs differently depending on, for instance, sensitization and desensitization processes. A sensitization of PMNGs to serum-opsonized zymosan, possibly mediated by C3 receptors or activated complement, has previously been observed *in vitro* and in exudate cells [52, 54–56]. Another possibility for the diverse effects of the drugs on leucocyte accumulation and CL could be that there were different concentrations of drugs in the two different locations (vascular versus chamber exudate).

In a recent study in rat muscle the NSAID diclofenac incorporated in a hydrogel reduced the number of PMNGs and macrophages surrounding the hydrogel [57]. Moreover, the distribution of cells in relation to the implant was altered. In this study the quantitative analysis was based on the retrieval of cells from a chamber. This chamber model does not give any firm soft tissue attachment to the outer surface of the chamber. Thus, a fluid space is established outside the chamber, allowing micromovements between the chamber surface and the surrounding soft tissue. Recent experiments indicate that the fluid space around the hollow implant contains similar types of cells as retrieved from the inside of the chamber (unpublished data). Although this free communication exists, we do not know whether betamethasone and indomethacin may have influenced the distribution of cells, that is the migration of leucocytes into the tissue between the fluid space and surrounding muscle.

Several factors related to methodology have to be considered when comparing the present data with those from other studies, including species, animal age, experimental models, drug concentrations and mode of administration. In addition, anaesthetic agents

(such as nembutal and apozepam) and solvents for drugs (such as DMSO) might influence the inflammatory reaction [48, 58, 59]. In earlier *in vitro* experiments nembutal and apozepam showed a dose-dependent inhibition of rat PMNG serum-opsonized zymosan-induced CL (unpublished data). We can therefore not exclude that the observed leucocyte accumulation and CL in the present study were influenced by the anaesthetic agents. However, control rats were injected daily with DMSO–HBSS in the same concentration as that given to treated rats and anaesthetic drugs were administered at surgery and killing in both treated and control groups.

This study shows the possibility of modulating the accumulation of inflammatory cells and their activity around an implanted biomaterial by daily systemic treatment with betamethasone and indomethacin. It is therefore possible that anti-inflammatory drugs via the interaction with cells in the exudate could also modify the levels of inflammatory mediators and different growth factors in the exudate, and possibly the long-term healing of an implanted material. The model might be a useful technique for local administration of various substances and, since the bottom part of the hollow implant is exchangeable, may be used for studies on the cellular response and healing of different implant materials during pharmacological treatment.

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