Selective Neutralization of Prostaglandin E₂ Blocks Inflammation, Hyperalgesia, and Interleukin-6 Production In Vivo

By Joseph P. Portanova,* Yan Zhang,* Gary D. Anderson,* Scott D. Hauser, Jaime L. Masferrer,* Karen Seibert,* Susan A. Gregory,* and Peter C. Isakson*

From the *Department of Inflammatory Diseases Research and †Department of Cell and Molecular Biology, G.D. Searle & Co., St. Louis, Missouri 63198

Summary

The role of prostaglandin E₂ (PGE₂) in the development of inflammatory symptoms and cytokine production was evaluated in vivo using a neutralizing anti-PGE₂ monoclonal antibody 2B5. In carrageenan-induced paw inflammation, pretreatment of rats with 2B5 substantially prevented the development of tissue edema and hyperalgesia in affected paws. The antibody was shown to bind the majority of PGE₂ produced at the inflammatory site. In adjuvant-induced arthritis, the therapeutic administration of 2B5 to arthritic rats substantially reversed edema in affected paws. Anti-PGE₂ treatment also reduced paw levels of IL-6 RNA and serum IL-6 protein without modifying tumor necrosis factor RNA levels in the same tissue. In each model, the antiinflammatory efficacy of 2B5 was indistinguishable from that of the nonsteroidal antiinflammatory drug indomethacin, which blocked the production of all PGs. These results indicate that PGE₂ plays a major role in tissue edema, hyperalgesia, and IL-6 production at sites of inflammation, and they suggest that selective pharmacologic modulation of PGE₂ synthesis or activity may provide a useful means of mitigating the symptoms of inflammatory disease.
neutralization were equivalent to those obtained in animals treated with the NSAID indomethacin. These findings demonstrate that PGE$_2$ is an important mediator of vascular permeability, hyperalgesia, and IL-6 production in vivo and they suggest that NSAIDs suppress certain inflammatory responses by inhibition of PGE$_2$ synthesis.

**Materials and Methods**

*Carrageenan-induced Edema and Hyperalgesia.* The induction of paw inflammation with carrageenan was performed as previously described (18, 21). Paw edema and hyperalgesia were induced by the injection of 0.1 ml of a 0.1% carrageenan solution in sterile saline (FMC Corp., Rockland, ME) into the hind footpad of a 200-g male Sprague Dawley rat (Charles River Laboratories, Por- tage, ME). The contralateral paw of each animal was injected with saline and served as the normal control. Paw swelling was measured with a water displacement plethysmometer at selected times after carrageenan injection. The edema response is expressed as the difference in paw volume between the carrageen- an- and saline-injected paws of each animal.

A hyperalgesic response to thermal stimulation was determined in the same animals by the method of Hargreaves et al. (22). Hind paws were exposed to radiant heat emitted from a high intensity projection bulb at selected times after injection. The amount of time in which each hind paw remained in contact with the heat source was measured to the nearest 0.1 s. The hyperalgesic response is expressed as the difference in the latency withdrawal period between carrageenan- and saline-injected paws of each animal.

In certain experiments, rats were administered indomethacin by oral gavage in 0.5% Methocel/0.025% Tween 80 (Sigma Chemical Co., St. Louis, MO) 1 h before carrageenan. Other rats were injected intraperitoneally with mouse ascites fluid containing the mouse anti-PGE$_2$ mAb, 2B5 or isotype-matched MOPC21 IgG1 myeloma protein (Sigma Chemical Co.) 18 h before carrageenan injection. The concentration of mouse IgG in ascites fluid was determined by a sandwich ELISA using species-specific anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) as the capture and detecting reagents, as previously described (20). The ELISA was also used to quantitate the circulating level of 2B5 in the plasma of passively immunized rats.

The carrageenan model of paw edema was modified by the method of Higgs et al. (23) to evaluate the inflammatory activity of exogenously administered PGs. Briefly, rats received a 30-mg/kg oral dose of indomethacin to prevent production of endogenous PGs. 1 h later, rats were injected in the hind footpad with carrageenan or with carrageenan plus 100 ng of PGE$_2$ or PGF$_2$ (Bio- mol, Plymouth Meeting, PA). Paw edema was then measured over a 3-h period to evaluate the ability of individual PGs to reconstitute the swelling response.

*Adjuvant Arthritis.* Arthritis was induced in male Lewis rats (Harlan, Indianapolis, IN) by footpad injection of Mycobacterium butyricum in mineral oil (Difco Laboratories, Detroit, MI) as described previously (24, 25). Dexamethasone and indomethacin (Sigma Chemical Co.) were suspended in Methocel/Tween and administered twice daily by gavage at dosages of 0.1 and 2 mg/kg, respectively. 2B5 and MOPC21 ascites were administered daily at a dose of 10 mg/kg by intraperitoneal injection. Treatments were initiated on day 15 postadjuvant injection and continued until final assessment of paw volume of un.injected contralateral paws on day 21. Previous results showed substantial paw swelling by day 15, as well as maximal levels of COX-2 protein and PGE$_2$ in arthritic paws (25).

**Eicosanoid Analysis.** PG and thromboxane levels in paw exudates were measured by ELISA (Cayman Chemical Co., Ann Arbor, MI). To facilitate the recovery of eicosanoids from paw tissue, paws were removed from rats after euthanasia with CO$_2$ and injected immediately with 0.1 ml of saline containing 30 $\mu$M indomethacin. Paws were centrifuged at 1,200 g, and the expressed fluid was analyzed for eicosanoids by ELISA.

**Cytokine Analysis.** Paws of selected animals were removed on day 21 to evaluate the effects of pharmacologic inhibitors and 2B5 on IL-6 and TNF RNA production. Total RNA was extracted from frozen and pulverized paws and hybridized to $^{35}$P-antisense probes complementary to rat IL-6 and TNF mRNA sequences as previously described (18, 25). Cytokine RNA levels were quantified by nuclease protection assay using an NPA II kit (Ambion Inc., Austin, TX), as described by the manufacturer. RNase digests were fractionated on an 8% polyacrylamide gel containing 7.5 M urea and the relative intensities of RNA fragments were determined using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Constitutive levels of GAPDH RNA were measured in parallel to correct for differences in sample loading among treatment groups. IL-6 protein levels in rat serum were estimated by a 7TD1 cell bioassay, as previously described (25).

**Results**

**Pharmacokinetics of 2B5 in Rats.** To determine the time that is required to achieve maximal plasma levels of 2B5 in the rat, the concentration of 2B5 was measured in peripheral blood at selected times after a single intraperitoneal injection of antibody. 2B5 was detectable in the plasma of recipient animals as early as 1 h after injection, and peak levels of 60 $\mu$g/ml were attained within 6 h (Fig. 1). 2B5

**Figure 1.** Time course of 2B5 absorption in rat plasma. The circulating level of the mouse anti-PGE$_2$ mAb 2B5 (lgGl.K) was measured in rat plasma at indicated times after injection. 2B5 was administered in the form of ascites fluid to Sprague Dawley rats ($n=6$) as a single 5 mg/kg dose by intraperitoneal injection. The concentration of mouse IgG in rat plasma was determined by a sandwich EIA using anti-mouse IgG antibodies that demonstrated negligible cross-reactivity with rat Ig.
continued to circulate at high levels when paw inflammation was induced 12 h later.

Effect of 2B5 on Acute Paw Inflammation. To examine the role of PGE\(_2\) in acute inflammation, we evaluated the effect of 2B5 in a rat model of carrageenan induced paw edema. Local injection of carrageenan resulted in the synthesis of multiple COX products in injected paws (Fig. 2). Enhanced amounts of PGE\(_2\), 6-keto PGF\(_{1\alpha}\) (the inactive metabolite of PGI\(_2\)), and thromboxane B\(_2\) were detectable in paw exudates within 1 h after injection; within 3–5 h, each eicosanoid increased maximally three to fivefold over baseline. In agreement with previous studies (16, 18), PG and thromboxane synthesis were prevented by indomethacin or a COX-2 selective inhibitor, SC-58125, administered 1 h before carrageenan stimulation (not shown).

The kinetics of local eicosanoid production coincided with the development of edema in the carrageenan-injected paw. The small increase in paw volume that occurred during the first h before PG production was not reduced by indomethacin (Fig. 3). This result agrees with previous findings showing that bradykinin and histamine are among the first mediators detected in inflammatory exudates after carrageenan stimulation (26). In contrast, the substantial increase in paw swelling that developed after the first hour was blocked by NSAID treatment, indicating a major role for PGs produced during the latter phase of the edema response.

The pharmacologic attenuation of paw edema by indomethacin was also observed in rats treated with the anti-PGE\(_2\) antibody (Fig. 3). 2B5 was administered 18 h before carrageenan to allow maximal distribution of antibody before induction of paw inflammation. Under these conditions, a 10 mg/kg dose of 2B5 was sufficient to completely prevent the NSAID-sensitive component of the edema response that occurred 1–3 h after carrageenan injection. The inhibitory effect of 2B5 appeared specific for PG-dependent inflammation, since the minor swelling that is insensitive to NSAID treatment was not affected. A MOPC21 control protein, matched for isotype and concentration, had no effect on paw edema at any time point.

Reconstitution of Paw Edema with Exogenous PG. A major role for PGE\(_2\) in edema formation was further supported by experiments in which the attenuation of paw edema by indomethacin was completely reversed by the addition of exogenous PGE\(_2\) to the carrageenan stimulus. As shown in Fig. 4, the magnitude of paw swelling induced in normal rats by carrageenan could be reproduced in indomethacin-treated rats that were injected with carrageenan supplemented with purified PGE\(_2\). Not surprisingly, edema occurred more rapidly in paws injected with carrageenan plus PGE\(_2\) than in paws in which PGE\(_2\) was induced after carrageenan injection. Exogenous PGI\(_2\) also potentiated the edema response to carrageenan, but was less effective than

Figure 2. Carrageenan induced eicosanoid production in rat paws. COX products in paws of Sprague Dawley rats injected locally with 1% carrageenan or saline were measured by ELISA at indicated times after injection. Each data point represents mean nanograms of eicosanoid/paw ± SE for six animals/group.

Figure 3. 2B5 and indomethacin reduce carrageenan-induced paw edema. Sprague Dawley rats were treated with a 10 mg/kg dose of 2B5 (■) or MOPC21 protein (□) by intraperitoneal injection for 18 h or with a 30 mg/kg oral dose of indomethacin (◇) or vehicle (●) for 1 h before injection of 0.1 ml of 1% carrageenan in saline into the hind paw. The difference in paw volume (mean ± SE) between carrageenan- and saline-injected paws determined 1–3 h later is presented (n = 6).
PGE₂. Preadministration of 2B5 to indomethacin-treated animals reduced the swelling in paws injected with carrageenan-PGE₂ stimulus to that elicited by carrageenan alone (Fig. 5). In contrast, 2B5 had no effect on paw swelling induced by carrageenan-PGI₂ under similar conditions. Taken together, these results underscore the importance of PGE₂ as a mediator of vascular permeability and edema formation at sites of inflammation.

Effect of 2B5 on Hyperalgesia. In addition to edema, carrageenan induced a hyperalgesic response in paws subjected to a thermal stimulus (Fig. 6). In agreement with previous studies (18, 22), the injection of carrageenan into the footpad reduced the time period (withdrawal latency) in which the inflamed paw remained in contact with a heated surface compared to the saline-injected paw (2.5 ± 0.4 s vs. 11.8 ± 0.6 s, respectively). This hyperalgesia was typically inhibited 60–80% in rats pretreated with a maximally effective dose of indomethacin (30 mg/kg) administered 1 h before carrageenan injection. Strikingly, the intraperitoneal administration of 2B5 (10 mg/kg) also reduced the hyperalgesic response ~80%. Neither 2B5 nor indomethacin extended the withdrawal latency of control paws, suggesting that the analgesic effect resulted from a peripheral rather than central mode of action. These results show that 2B5 and indomethacin were equally efficacious in attenuating hyperalgesia associated with inflammation, and they further demonstrate the importance of PGE₂ in potentiating pain at the inflammatory site.

Dose Response Analysis of 2B5 and Indomethacin. Dose response studies indicated that 2B5 prevented edema and hyperalgesia in a concentration-dependent manner. A 10 mg/kg dose of 2B5 caused maximal inhibition of both responses, and half maximal effects were obtained with ~1 mg/kg of antibody (Fig. 7 A). The coincident nature of the 2B5 dose–response curves suggests that the effective concentration of PGE₂ necessary to induce edema and hyperalgesia is similar. Interestingly, the maximal reduction of both responses by 2B5 was comparable to that obtained with optimal doses of indomethacin (Fig. 7 B).

2B5-mediated Neutralization of PGE₂. To determine whether 2B5 bound PGE₂ in vivo, we measured the amount of free PGE₂ in paw exudates of antibody-treated animals by ELISA. The data presented in Fig. 8 show the characteristic rise in PGE₂ levels in paws injected 3 h previously with carrageenan, as well as the inhibition of PGE₂ production by indomethacin. Paw levels of free PGE₂ were also reduced to baseline in rats treated 18 h earlier with 10 mg/kg of 2B5, a dose that prevented the PG-dependent increase in paw edema and hyperalgesia. An equivalent dose of MOPC21 did not alter PGE₂ levels in inflamed paws. The ability of 2B5 to reduce the levels of free PGE₂ induced by carrageenan to those in normal paw tissue strongly suggests that the antiinflammatory effect of 2B5 resulted from neutralization of PGE₂ at the inflammatory site.

Effect of 2B5 on Paw Inflammation in Adjuvant Arthritis. Since NSAIDs reverse paw edema and IL-6 production associated with adjuvant arthritis (24, 25), we determined the extent to which 2B5 could produce similar antiinflammatory effects when administered therapeutically to arthritic rats. The data presented in Fig. 9 show that the injection of mycobacteria into the right hind footpad caused a 1-ml increase in the volume of arthritic contralateral paws compared to normal controls 14 d after adjuvant injection. At this time, arthritic rats were segregated into groups and treated daily with indomethacin, 2B5, or dexamethasone for 7 d. As observed in our previous study (25), significant
reversal of established edema by indomethacin and dexamethasone was observed by day 18, and maximal effects were attained by day 21.

Paw swelling in arthritic rats was also reversed by therapeutic administration of 2B5. A 10 mg/kg daily dose of 2B5 on days 18 and 21 was nearly identical to that obtained by blockade of PG synthesis with indomethacin. Neither agent was as effective as dexamethasone, which blocks the production of multiple inflammatory mediators in addition to PGs. Paw swelling remained suppressed in indomethacin-treated rats through day 25, yet it reappeared in 2B5-treated animals (not shown). This loss of antiinflammatory activity was associated with a rat antibody response to the injected mouse antibody.

Anticytokine Effects of 2B5. We also examined the effect of 2B5 on the production of the inflammatory cytokines IL-6 and TNF in adjuvant arthritis. As shown in Fig. 10 A, indomethacin and dexamethasone significantly blocked the substantial accumulation of IL-6 mRNA in arthritic paws 21 d after adjuvant injection. The inhibitory effect of both drugs was also manifested systemically, since serum levels of IL-6 protein were reduced to normal levels (Fig. 10 B). In contrast to IL-6, TNF mRNA levels were reduced only by dexamethasone, indicating a differential regulation of cytokine production by PGs (Fig. 11). An increase in TNF mRNA was observed in one of three indomethacin-treated animals presented in the figure; however, this finding was not representative of results obtained in three other experiments (not shown).

We used 2B5 to address the role of PGE2 in IL-6 mRNA and protein production in this model. Data in Fig. 10 B show that the therapeutic administration of 2B5 to arthritic rats beginning on day 14 caused a marked reduction in IL-6 RNA levels in arthritic paws and serum levels of IL-6 protein measured 21 d after adjuvant injection. The inhibitory effect, however, was less than what was achieved with indomethacin, suggesting that other PGs may also be involved in regulating IL-6. In contrast, TNF mRNA levels in the same paws were not modulated by 2B5, indicating the antibody did not reduce total mRNA levels in the inflamed tissue (Fig. 11).

Discussion

Global inhibition of PG synthesis by NSAIDs is a widely used treatment of inflammatory symptoms associated with rheumatoid arthritis and other immunoinflammatory dis-
Figure 9. Therapeutic attenuation of paw edema by indomethacin, dexamethasone, and 2B5 in adjuvant arthritis. Arthritis was induced in male Lewis rats by injection of M. butyricum in oil into the right hind footpad, as described in Materials and Methods. On day 14 after adjuvant injection, rats were segregated into groups and treated daily with dexamethasone (0.1 mg/kg, bid, oral), indomethacin (2 mg/kg, bid, oral), 2B5 or MOPC21 (10 mg/kg i.p., once per day) for 7 d. The volume of contralateral (uninjected) arthritic paws was determined by plethysmometry on days 14, 18, and 21. Paw volume of normal littermates was measured as controls. Data are presented as mean paw volume ± SE, n = 6/group. The results are representative of three independent experiments. --, normal; ---, adjuvant; △, indomethacin; □, 2B5; ○, MOPC21; △, dexamethasone.

Figure 10. Inhibition of adjuvant-induced IL-6 RNA and protein by dexamethasone, indomethacin, and 2B5. (A) Nuclease protection assay. Contralateral paws of adjuvant-injected rats were recovered on day 21 after adjuvant injection for RNA extraction and analysis. Autoradiogram of IL-6 RNA (three paws/group) is shown above the mean relative intensities determined by phosphoimager analysis of respective bands. The level of GAPDH RNA detected by nuclease protection was similar in all samples (not shown). (B) IL-6 bioassay. Serum was collected from Lewis rats 21 d after adjuvant injection for IL-6 quantitation. IL-6 bioactivity (U/ml) was determined by support of proliferation of murine 7TD1 cells and extrapolation from a standard curve generated with mouse rIL-6. Data are presented as mean U/ml ± SE, n = 6/group.
ane are also produced in these inflammation models (16, 18, 19, 25), our passive immunization data suggest that PGE₂ appears necessary for the full and sustained expression of edema and hyperalgesia in affected tissue. This conclusion is substantiated by reconstitution experiments in which exogenous PGE₂, but not PGI₂, fully restored edema formation in carrageenan-challenged rats that were depleted of endogenous PGs with indomethacin. Ferreira et al. (9-11) similarly showed that while infusion of purified PGE₂ or PGI₂ elicited hyperalgesia in rodents and man, the effects of PGE₂ were sustained for several hours while those of PGI₂ were short lived. Our results suggest that analogous studies will help define the contribution of endogenous PGI₂ to edema and hyperalgesia. In this regard, Smith et al. (29) have previously demonstrated the effectiveness of polyclonal antibodies in neutralizing the hypotensive activity of PGI₂ in vivo.

In addition to addressing the role of PGE₂ in promoting inflammatory symptoms, we examined the effect of 2B5 on inflammatory cytokine production in rat adjuvant arthritis. Our previous study showed that the marked upregulation of IL-6 RNA production in this model was inhibited by COX-2 inhibitors (25). Passive immunization experiments with 2B5 extend these findings by implicating PGE₂ as a critical regulator of IL-6 production in adjuvant arthritis. We observed that the therapeutic treatment of rats with 2B5, but not MOPC control protein, significantly reduced IL-6 RNA expression in arthritic paws and IL-6 protein levels in serum. These findings are consistent with the ability of PGE₂ and other cAMP-inducing agents to upregulate cellular levels of IL-6 RNA in vitro (30, 31).

In contrast to its positive effect on IL-6 synthesis, PGE₂ may downregulate TNF RNA and protein synthesis by monocytes and lymphocytes in vitro (32, 33). A similar in vivo role for PGE₂ has been suggested by the enhancing effect of NSAIDs on TNF production in LPS-challenged mice (34, 35). However, we failed to reproducibly observe enhanced levels of TNF RNA in the paws of indomethacin- or 2B5-treated rats 21 d after adjuvant injection. Similar findings were obtained in the analysis of TNF RNA in animals with late-stage adjuvant arthritis that were treated with COX-2 inhibitors (25). This apparent discrepancy in the role of PGE₂ in regulating TNF in various inflammation models may relate to differences in inflammatory stimuli, as well as the acute vs. chronic nature of the inflammatory responses. It is plausible that PGE₂ may downregulate TNF production in the early stages of the inflammatory response, whereas its effect may become less important as other mediators are released later in the disease process.

Considerable evidence indicates that the inflammatory effects of PGE₂ are mediated through G protein-coupled EP receptors on cell plasma membranes (27, 36). Recently, an additional mechanism has been proposed in which PGs may exert their biologic effects directly within the nucleus (36). Studies by Smith et al. have identified the COX-2 enzyme in functionally active form in association with the nuclear membrane (37-39). In addition, other enzymes such as cytosolic phospholipase A2 have been shown to translocate to the nuclear membrane where arachidonic acid is released as a substrate for COX-2-mediated PG synthesis (40). These intriguing observations have raised the possibility that newly synthesized PGs may act directly within the nucleus in addition to activating G protein-coupled receptors on the cell surface. While NSAIDs would be ineffective in distinguishing the relative contribution of these signaling pathways, it is likely that neutralizing mAbs...
would bind to and neutralize the activity of extracellular PG without affecting PG acting within the cell. Consequently, the antiinflammatory properties of 2B5 described herein suggest that potentiation of edema, hyperalgesia, and IL-6 production requires an interaction of extracellular PGE₂ with cell-surface receptors. Whether signals transduced by PGE₂ through EP receptors are sufficient to potentiate edema and hyperalgesia, or whether additional signals are transduced through nuclear PGs, remains an interesting subject for future experimentation.

Address correspondence to Joseph Portanova, G.D. Searle & Co./AASG, 700 Chesterfield Parkway North, St. Louis, MO 63198.

Received for publication 7 November 1995 and in revised form 30 May 1996.

References


