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Review

Antimicrobial activity of non-steroidal anti-inflammatory drugs with respect to immunological response: Diclofenac sodium as a case study

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The non-steroidal anti-inflammatory drugs (NSAIDs) as the name implies are compounds of non-steroidal origin, with the capability of inhibiting/reducing inflammatory response associated with tissue injury which could be as a result of physical trauma, noxious chemicals or microorganisms. There is however reason to believe that these drugs, which confound disease progression by suppressing fever, pain and attenuating some of the cardinal manifestations of inflammation in a patient actually lessen the immunologic response to bacterial infection. This seemingly paradoxical property is the birth of this mini review. This review aims at invalidating the claims that NSAIDs in general lessen the immunological response to microbial infections by examining the antimicrobial properties of diclofenac sodium, an NSAID.

Key words: Immunologic response, inflammation, NSAIDs, Diclofenac, biofilms.

INTRODUCTION

Increased interest in NSAIDs, traditionally known as the analgesic-antipyretics came with the discovery of their profound anti-inflammatory properties. The discovery afforded the opportunity to have a single drug therapy that would alleviate the problems of pain, fever and inflammation frequently associated with many underlying disease conditions. In the last decade, there has been renewed interest in NSAIDs and surprisingly this does not stem from their traditional properties or uses, but as a result of new properties, which they may possess. Inflammation is a body's normal protective response to tissue injury. It is the body's effort to inactivate or destroy invading microorganisms, remove irritants and set the stage for tissue repair. When healing is complete though, the inflammatory process usually subsides (Mycek et al., 2000). Sometimes however, the defense reactions themselves cause progressive tissue injury as in the case of arthritis requiring anti-inflammatory (or immunosuppressive) drugs to modulate the inflammatory process (Mycek et al., 2000; Payan and Katzung, 1995). Also local and systematic inflammations such as those elicited by bacterial infections at mucosal surfaces are often times treated with NSAIDs in combination with other drugs. However, the use of anti-inflammatory therapy has been questioned on the basis that such treatment may compromise the immune function. Several reports have implicated NSAIDs as having actions that may lessen immunological response to bacterial infections (File, 2003; Linder et al., 1990; Stevens, 1995) and as such while confounding the progression of disease by suppressing inflammation, fever and pain, they could actually be enhancing the progression of bacterial infection.

THE INFLAMMATORY RESPONSE

Inflammation is a general, non-specific reaction to foreign particles and other noxious stimuli such as toxins and pathogens. It is the body's effort to inactivate or destroy

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invading organisms, remove irritants and set the stage for tissue repair (Madigan et al., 2000). Characteristics of the inflammatory response include redness, swelling, pain and heat which are localized at the site of infection (Furr, 1992). Even though inflammation is a non-specific immunological reaction, it employs specific cell mediated immunological responses such as the leukocytes and their cytokine secretions, which mediate on inflammatory reactions. Inflammation is triggered by the release of chemical mediators from injured tissues and migrating cells. The specific chemical mediators vary with the type of inflammatory process and include amines e.g. histamines and 5-hydroxytryptamine lipids e.g. prostaglandins, small peptides such as bradykinin and larger peptides such as interleukin-1, which is a cytokine (Furr, 1992; Mycek et al., 2000). Most NSAIDs act by inhibiting the synthesis of prostaglandins. Thus, understanding of NSAIDs require a comprehension of the actions of prostaglandins.

Prostaglandins (PGs) and related fatty acid derivatives of arachidonic acid are among the most potent naturally occurring autacoids. They are critically important cell regulatory substances (Rang et al., 1999). The PGs and several other biologically active lipids and peptidolipid acids are formed from the same precursor (arachidonic acid) through interrelated enzymatic pathways. These other lipids are nearly all carboxylic acids and include the thromboxanes (TXs), the hydroxyeicosatetraenoic acids (HETEs), the leukotrienes (LTs), most recently discovered lipoxins (LXs) and epoxyeicosatetraenoic acids (EETEs). They are generally known as the Eicosanoids (Hecker et al., 1995). Free arachidonic acid is metabolized mainly by two divergent enzymatic pathways that are variably distributed among different cells: the cyclooxygenase (COX) and the lipoxygenase pathways (Mycek et al., 2000; Payan and Katzung, 1995; Rang et al., 1999). The products of the cyclooxygenase pathway are the ring structured eicosanoids (PGs, TXs and Prostacyclines) catalyzed by two enzymes COX-1 and COX-2. The COX-2 appears to be the form of the enzyme associated with cells involved in the inflammatory process. The COX-1 is a constitutive enzyme expressed in most tissues including blood platelets. It is involved in cell-cell signaling and in tissue homeostasis (Rang et al., 1999). Clearly, the anti-inflammatory action of NSAIDs is mainly related to their inhibition of COX-2. Most of the undesirable effects of NSAIDs have been suggested to result from their inhibition of the COX-1 enzymatic pathway (Payan and Katzung, 1995). The lipoxygenase pathway is catalyzed mainly by 5-lipoxygenase enzyme. The products of this pathway are the 5-HPETE, 12-HPE TE and HPETE, which are unstable and converted to the HETEs or to leukotrienes or lipoxins depending on the tissues (Mycek et al., 2000).

Prostaglandins and related compounds are produced in minute quantities by virtually all tissues. They generally act on the tissues in which they are synthesized and are rapidly metabolized into inactive products at their sites of action. Thus, the PGs do not circulate in the blood in significant concentrations (Mycek et al., 2000). In general, prostaglandins have a variety of effects on smooth muscles, platelets, reproductive system, central nervous system and on cells involved in inflammation. The leukotrienes are present in the tissues in many inflammatory conditions. They have a powerful chemotactic effect on eosinophils, neutrophils and macrophages. They promote broncho-constriction and alter vascular permeability. The prostaglandins are not chemo-attractants, but the leukotrienes and some of the HETEs are strong chemo-attractants (Hecker et al., 1995).

The primary inflammatory cytokines are important co-players when COX-2 is induced in inflammatory cells. They regulate cyclooxygenase and lipoxygenase enzymatic activities (Hecker et al., 1995; Rang et al., 1999b) hence they shall be discussed briefly. As mentioned earlier, there are other chemical mediators that play important roles in the inflammatory processes. Their discuss, however, is outside the scope of this work. The cytokines are another group of chemo-attractants produced by leukocytes. They are, by nature, proteins and are responsible for recruiting and activating non-specific effector cells such as the phagocytes (Madigan et al., 2000). They are released from inflammatory tissues, connective tissues and immune system cells. They act by autocrine and paracrine mechanisms in a manner somewhat similar to hormonal actions. In most instances, cytokine effects are modulated via cell surface receptors on target cells. Cytokines are known to have anti-proliferative and antimicrobial activities (Salmon et al., 1995). For example, infections with Gram negative [G-ve] bacteria induce inflammatory response that could be local or systemic (De Man et al., 1988; Gilbert, 1992). The inflammation is elicited by whole bacterium and lipid A moiety of lipopolysaccharide (LPS) on the surfaces of G-ve bacteria (De Man et al., 1989; Hagbery et al., 1983; Rietschel et al., 1982). The LPS induces the recruitment of a wide range of cytokines, such as the tumour necrosis factor, (TNF), Interleukin-1 (IL-1), IL-6 and colony stimulating factors (CSFs) (Dinarello et al., 1988; Nijsten et al., 1987; Perimutter et al., 1986). More than 50 cytokines have been identified to date and their super families include interleukins, chemokines, colony-stimulating factors, growth factors, interferons, transforming growth factors (TGF) and the tumour necrosis factors (TNFs). They have varied actions. Some are pro-inflammatory cytokines and include the TNF and interleukin-1 (IL-1). The anti-inflammatory cytokines include TGF-B, IL-4, IL-10 and IL-13.

The inflammatory response is a reflection of the severity of disease (De Man et al., 1989). It can be quantitated by the recruitment of the acute phase reactants, pyrogens, chemoattractants such as cytokines and the polymorphonuclear leukocytes (PMNL) (Linder et al., 1990; Shahin et al., 1987). The assay of IL-6 as a measure of

inflammation is particularly preferred though, this is based mainly on the accuracy of the assay. The hybridoma assay for IL-6 has a high degree of specificity (De Man et al., 1989). In contrast, some commonly used assays such as the human-urine IL-1 have had their validity being debated over (Helle et al., 1988; Nelle et al., 1988).

THE ROLE OF NSAIDS IN THE INFLAMMATORY RESPONSE

As earlier mentioned, NSAIDs act by blocking prostaglandin and thromboxane synthesis. This is achieved through the inhibition of the cyclooxygenase pathway which is responsible for their synthesis. With a few exceptions, NSAIDs do not inhibit lipoxygenase activity at concentrations that markedly inhibit cyclooxygenase activity (Hecker et al., 1995). Since the lipoxygenase and cyclooxygenase pathways have the same precursor (arachidonic acid), inhibiting the metabolism of arachidonic acid via the cycloxygenase pathway would cause metabolism to tend more to the lipoxygenase pathway; thus causing more products formation than usual via that pathway. Hence, the use of NSAIDs leads to an increase in the formation of inflammatory leukotrienes. It has been discovered that even among the cyclooxygenase dependent pathways, inhibiting the synthesis of one derivative may increase the synthesis of an enzymatically related product. This is however not usually desirable as the thromboxanes and leukotrienes are known to be 'pathologic eicosanoids' (Hecker et al., 1995). The activities of prostaglandins and leukotrienes in the body produce somewhat opposite effects. Prostaglandin-E_2 (PGE_2) and PGI_2 are the two common prostaglandins synthesized in humans. PGE_2 inhibits the activation and proliferation of B-lymphocytes by T-lymphocytes ($CD4^+$) while leukotriene-B_4 (LTB_4) stimulates it while inhibiting both antigen-driven and mitogen-induced B-lymphocyte proliferation and differentiation to plasma cells. This results in the inhibition of immunoglobulin M (IgM) synthesis and enhanced class switch to IgE. PGE_2 and possibly PGI_2 inhibit the expression of IL-1 and its effect on T-lymphocytes, while LTB_4 and LTD_4 increase its expression. PGE_2 and probably PGI_2 inhibit interferon gamma (INT-γ) activity on macrophages, while LTB_4, LTC_4 and LTD_4 stimulate it. Also PGE_2 and possibly PGI_2 inhibit the action of IL-2 from $CD4^+$ lymphocytes to $CD8^+$, LTB_4 does the reverse (Hecker et al., 1995; Salmon et al., 1995). It can thus be inferred that the blockage of the cyclooxygenase pathway leads to the increased activity of the lipoxygenase pathway. Diclofenac and indomethacin have however been reported to reduce synthesis of both prostaglandins and leukotrienes (Payan and Katzung, 1995) and consequently the increased expression of the cytokines (Figure 1). The analgesic and antipyretic actions of NSAIDs are attributed to PGE inhibition. Also undesirable effects such as the ulcerative and decreased renal blood flow properties of NSAIDs are attributed to inhibition of PG synthesis (Mycek et al., 2000; Payan and Katzung, 1995). It should be mentioned however that corticosteroids have the unique ability of blocking simultaneously the cyclooxygenase and lipoxy-genase pathways. This is achieved by inhibition of the phospholipase activity (that is, the enzyme that catalyzes arachidonic acid synthesis). This would invariably inhibit both cyclooxygenase and lipoxygenase pathways.

THE ANTIBACTERIAL PROPERTY OF DICLOFENAC

In recent years, due to the increased resistance of many bacteria to the commonly used antimicrobial agents, attention has shifted to drugs belonging to different pharmacological classes for possible antimicrobial activity. This, borne out of the fact that a single drug may have varying properties with diverse physiological activities and functions and as a result, may have useful activities in completely different spheres of medicine. This, in turn, has led to the investigation of drugs belonging to different pharmacological classes for possible antimicrobial activity. At present, drugs from various pharmacological classes such as promethazine-an antihistamine, promazine-a psychotropic agent, methyledopa - a local anaesthetic, dobutamine -a cardiovascular drug and diclofenac, an NSAID, have been discovered to possess potent antimicrobial activities (Annaduri et al., 1998; Chakrabarty et al., 1989; Chattopadhyay et al., 1988; Dash et al., 1977; Dastidar et al., 1976; Dastidar et al., 1986; Dastidar et al., 2003) and are now referred to as "non-antibiotics" (Kristiansen, 1992).

In a trial experiment carried out in our laboratory on the *in vitro* antimicrobial activity of Diclofenac sodium using the bore-hole method on Mueller-Hinton agar (CM337 Oxoid), Diclofenac sodium was found to possess considerably good antimicrobial properties, on incubating for 24 h at 37˚C, as shown in Table 1. In other recorded studies, concentrations of diclofenac sodium (50-100 ug/ml) have been shown to possess *in vitro* activity against most bacteria (Annaduri et al., 1998). *In vitro* assays using 30 - 50 µ/20 g mouse have also been shown to possess appreciable antibacterial activity against virulent *Salmonella typhimurium* strains (Dastidar et al., 2000). Though much about the mechanism of activity is still not very clear, some have attributed it to be a result of the inhibition of DNA synthesis in bacterial cells (Dastidar et al., 2000). In this report the anti-bacterial action of Diclofenac was found to be due to the inhibition of DNA synthesis which was demonstrated using 2 µ Ci(3H) deoxythymidine uptake. In the light of this, could diclofenac join other drugs such as bleomycin and doxorubicin as anti-tumour antibiotics? Further work would have to be done to ascertain the possibility of this. It has however been proven to be a drug with great potential for use as an antimicrobial agent.

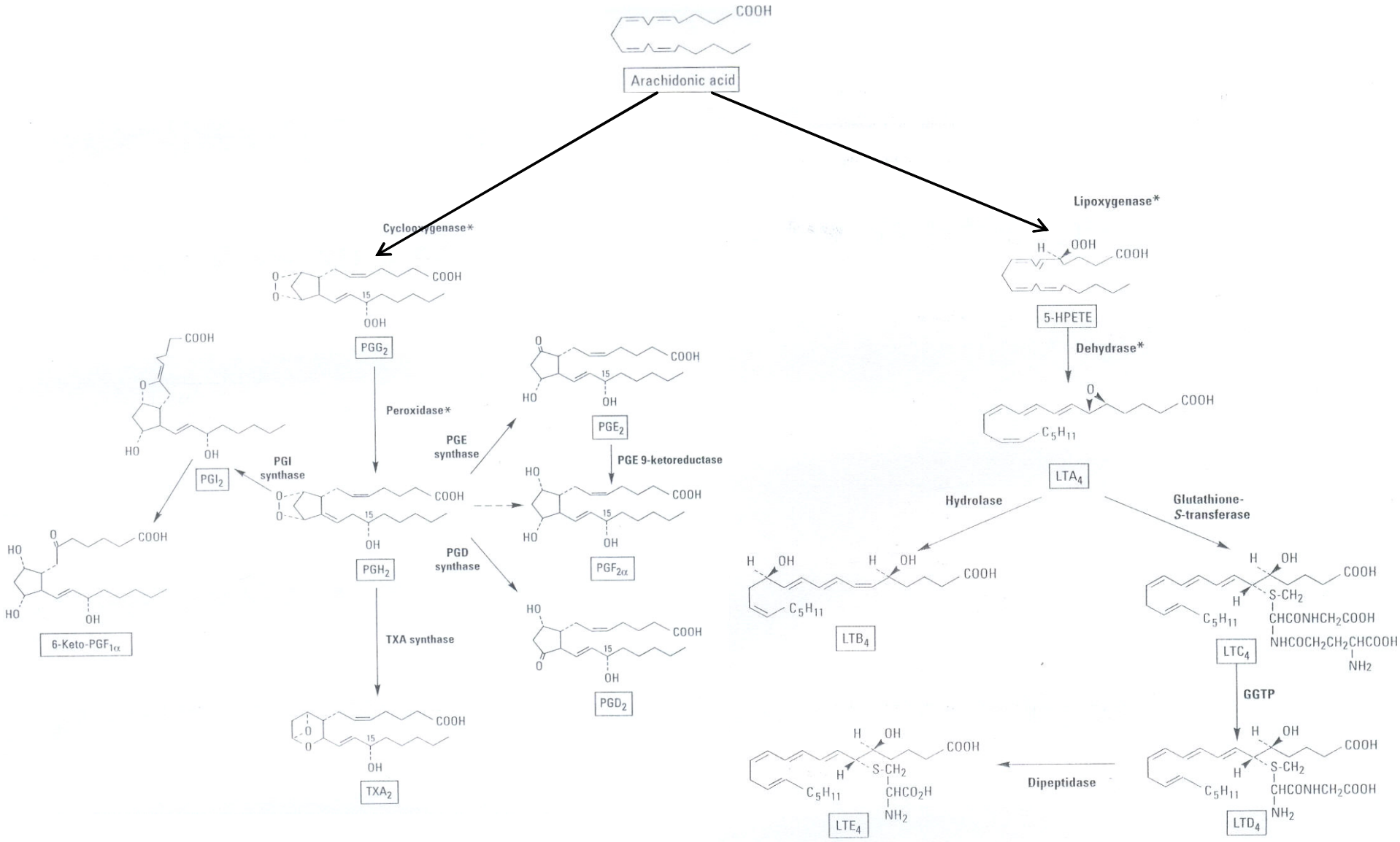

Figure 1. The funneling of substrate between the cyclooxygenase and lipoxygenase pathways. The cyclooxygenase pathway involves the prostaglandin and thromboxane biosynthesis. The asterisks for the cycooxygenase pathway indicate that the cyclooxygenase and peroxidase steps are catalyzed by a single enzyme, prostaglandin endoperoxide (PGH) synthase. The lipoxygenase pathway involves leukotriene biosynthesis. The asterisks in this pathway indicate that both the lipoxygenase and dehydrase reactions are driven by the single enzyme, 5-lipoxygenase (GGTP, γ-glutamyltranspeptidase (Modified from Foegh and Ramwell, 2001).

Table 1. *In vitro* antimicrobial activity of diclofenac sodium on some pathogenic microorganisms.

Organism	ATCC Strain	CFU/0.1 ml	Conc of Diclofenac Sodium/0.1 ml	Zone of Inhibition (mm)
E. coli	9637	10^6	2.5 mg	14 (cidal)
P. aeruginosa	27853	10^6	2.5 mg	8 (cidal)
S. typhi	13709	10^6	2.5 mg	13 (cidal)
C. albicans	1023	10^6	2.5 mg	12 (cidal)

ACTIVITY ON BIOFILMS

In addition to having antimicrobial properties, diclofenachas recently been discovered to inhibit microbial biofilms. A biofilm is a population of cells growing on a surface and enclosed in an exopolysaccharide matrix (Lewis, 2000; Lewis, 2001). Biofilms confer resistance on micro-organisms to antibiotic treatment (Brooun et al., 2000; Costerton et al., 1995; Douglas and Ballie, 1998; Gander, 1996). The development of resistance by microorganisms to antimicrobial drugs has been one of the greatest problems hampering antimicrobial therapy (Olaniyi, 1989). Bacterial biofilms show enormous levels of antibiotic resistance. In fact, resistance to antimicrobials is a general feature of all biofilms (Brooun et al., 2000; Douglas and Ballie, 1998). Biofilms are the major cause of recalcitrant infections and might be responsible for 65% of all bacterial infections (Brooun et al., 2000; Costerton et al., 1999; Potera, 1999). *Candida albicans*, a major fungal pathogen of humans (Cox and Perfect, 1993) has been discovered to form biofilms (Douglas and Ballie, 1998; Hawser and Douglas, 1994). Others include *Staphylococcus* spp, *Pseudomonas* spp and *Escherichia coli* (Aries et al., 1999; Brooun et al., 2000; Lewis, 2000; Lewis, 2001). Most susceptibility studies done on biofilms are on preformed biofilms and not on the ability of a biofilm to grow, hence antimicrobials reported to inhibit biofilm growth are those, which are able to diffuse through the preformed biofilms and exert their actions (Lewis, 2001). Examples include the fluroquinolones, cephalosporins, rifampicin. The problem with these measurements is that the results are not entirely conclusive. Functional tests for growth could be a better assay protocol for biofilm susceptibility to antibiotics.

In one experimental model, viability assays were carried out on both growing and fully mature (48 h cultures) biofilms to investigate the effects of aspirin, diclofenac and other NSAIDs on biofilm formation (Alem and Douglas, 2004). Results showed that diclofenac, aspirin and etodolac had the greatest inhibitory effect, with aspirin causing up to 95% inhibition. Celecoxib, nimesulide, ibuprofen and meloxican also inhibited biofilm formation, but to a lesser extent. The inhibition of prostaglandin synthesis was suggested to be the mode of action of NSAIDs on the inhibition of biofilm formation. On the other hand, Diclofenac was reported to inhibit significantly germ tube formation, in addition to inhibiting biofilm formation - a feature not expressed by aspirin (Alem and Douglas, 2004). Could this additional feature be a function of diclofenac's antimicrobial action on *Candida albicans* (Costerton et al., 1999)? Biofilms are resistant to the immune system as a result of their exopolysaccharides (Hoyle et al., 1990). It could then be inferred that Diclofenac would be a good therapeutic agent for treating persistent or relapsing bacterial and fungal infections. It is however recommended that further investigations be carried out on the inhibition of biofilm formation using other potent prostaglandin inhibitors such as corticosteroids, since the inhibition of prostaglandin synthesis was suggested (Alem and Douglas, 2004) to be NSAIDs' mechanism of inhibiting biofilm formation.

THE UNUSUAL SUSPECT

The use of NSAIDs has been previously perceived as one that would not alter patient responses to infection (Payan and Katzung, 1995). Bacterial infections induce inflammatory responses which sometimes require anti-inflammatory therapy. The use of NSAIDs is particulary favoured because unlike the corticosteroids which confound host immunity (Salmon et al., 1995), NSAIDs are generally perceived as not having actions that compromise immunity. This has greatly encouraged their use in bacterial induced inflammation and other inflammatory conditions which require long term treatment or in which host immunity is likely to be threatened by use of corticosteroids. Some more recent reports have however implicated NSAIDs as having actions that may enhance the progression of bacterial infections (Barnham and Anderson, 1997; Smith and Berk, 1991; Stevens, 1995). There is reason to believe that NSAIDs have several actions that may lessen the immunologic response to bacterial infections. That by their actions, such as impairment of granulocyte function (adherence, phagocytosis, cidal activity), augmentation of inflammatory cytokine release and inhibition of renal prostaglandin synthesis, NSAIDs confound the remission of disease (File, 2003). Reports have implicated NSAIDs as predisposing factors to bacterial infections (Stevens, 1995).

While augmentation of inflammatory cytokine release

(that is, inhibition of PG synthesis by NSAIDs) results in increased leukotriene synthesis, which in turn favours cytokine release (e.g. PGE_2 and PGI_2 have inhibitory effect on IL-1 production, while LTB_4 has reverse effect), it has, on the other hand, been suggested as a mechanism by which NSAIDs lessen the immunological response to bacterial infections (others have cited that the inflammatory response quantitated as the recruitment of PMNL and increased level of cytokines coincides with the clearance of infection) (File, 2003). These reports suggested that cytokine release and consequently inflammation is responsible for the elimination of bacteria and infection (Coulie et al., 1987). Further explanations suggest that the inhibition of inflammation impairs bacterial clearance from the kidneys. Suggestions have however been made that the impairment of bacterial clearance as a result of inhibition of inflammation is not a direct function of the inhibited cytokine (IL-6) or PMNL recruitment (Linder et al., 1990). Indomethacin (an NSAID) was experimented upon and found to reduce bacterial clearance (Linder et al., 1988). Similar experiments using diclofenac also produced a reduction in bacterial clearance, although the concentrations used to produce this effect were very high and lower (normal) concentrations did not reduce bacterial clearance (Linder et al., 1990). In another experiment on the influence of antiinflammatory therapy on bacterial clearance, the finding was that; following intramammary *E. coli* challenge in glands, non-steroidal anti-inflammatory therapy did not adversely influence the clearance of *E. coli* from challenged glands (Anderson et al., 1991).

In another development, diclofenac was reported to protect rabbits from necrotizing fasciitis caused by Group A *Streptococcus* (GAS) (Guibal et al., 1998). Reports have also shown diclofenac not to adversely affect therapy when used in combination with anti-tubercular drugs in treatment of *Mycobacterium avium* complex (MAC) (Sano et al., 1999). Moreover in this report, diclofenac in combination with the anti-tubercular drug used had higher clearance of MAC from the lungs although it was not significantly different ($p < 0.001$) from reduction in bacterial load brought about by the use of the anti-tubercular agent solely.

CAN A NON-STEROIDAL ANTI-INFLAMMATORY DRUG WITH ANTIMICROBIAL PROPERTIES, REDUCE IMMUNOLOGIC RESPONSE TO BACTERIAL INFECTIONS?

It is still not an established fact that NSAIDs lessen the immunologic response to bacterial infections. Though the anti-inflammatory drugs; dexamethasone (a corticosteroid), diclofenac and indomethacin (NSAIDs) have been reported to decrease bacterial clearance from kidneys of *E. coli* infected mice (Linder et al., 1990). Contrasting reports state that anti-inflammatory therapy did not adversely influence clearance of *E. coli* from challenged glands (Anderson et al., 1991) in goats. Reports based on experimental data have even suggested that the administration of diclofenac after infection protected rabbits from necrotizing fasciitis caused by Group A *Streptococcus* (GAS) and that diclofenac did not potentiate tissue damage (Guibal et al., 1998).

There is good reason to believe that anti-inflammatory therapy may compromise immune function and this is particularly true of the corticosteroids. They are known not only to greatly reduce inflammation, but also immune response. They decrease the concentration of lymphocytes (B and T cells), basophils, eosinophils, monocytes and bring about inhibition of the ability of leukocytes and macrophages to respond to mitogens and antigens (Mycek et al., 2000). Steroids relieve inflammation by inhibiting the synthesis of arachidonic acid and consequently its metabolism via both lipoxygenase and cyclooxygenase pathways. Thus, the action of steroids greatly reduces cell mediated and inflammatory responses (Salmon et al., 1995). NSAIDs on the other hand inhibit the metabolism of arachidonic acid only through the cyclooxygenase pathway. The lipoxygenase pathway is left functional and hence the production of leukotrienes and cytokine expression (Figure 1). A good reference to this has been discussed (Barnham and Anderson, 1997) where dexamethasone on administration was found to reduce both interleukin (IL-6) secretion and PMNL response while indomethacin was found not to decrease neither IL-6 secretion nor PMNL response. Diclofenac was found to decrease IL-6 levels in the urine, but not in serum (Barnham and Anderson, 1997). Diclofenac and indomethacin were found to significantly increase IL-6 levels after 24 h treatment (IL-6 secretion was reported to coincide with bacterial clearance) (Barnham and Anderson, 1997). With reference to Figure 1, it can be inferred that treatment with NSAIDs does not block the inflammatory immune response to bacterial infections. Diclofenac was mentioned not to decrease bacterial clearance at lower (therapeutic) but only at higher concentrations. These high concentrations are however exaggerated and may be toxic.

It should be mentioned that inflammation might actually promote bacterial growth. Inflammation leads to fluid accumulation in the area of injury due to increased permeability, this leads to localized oedema, which may actually promote bacterial growth (Fur, 1992). Another means by which it may aid microbial pathogenesis is borne out of the fact that the inflammatory response elicited by an invading organism can result in considerable host damage, making nutrients available and providing access to host tissues (Madigan et al., 2000), hence the necessity for anti-inflammatory therapy.

QUESTIONS ARISING

1) Do NSAIDs in general lessen immunologic response to

bacterial infections, or is it a property peculiar to some?
2) Do they decrease bacterial clearance or is this also peculiar to some?
3) Does the level of selective inhibition of COX-1 and COX-2 affect the immunologic response to infections?

The therapeutic benefits of having one drug as an analgesic, antipyretic, anti-inflammatory and an anti-microbial should be greatly explored. Further work would have to be performed to explore this potential.

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