concentrations of 0.1–100 μg/mL ranged from 95–97% in dog plasma, and the binding appeared to be similar in the plasma from the male and the female dogs. The binding was not concentration dependent, and saturation of the binding sites was not apparent at concentrations up to 100 μg/mL.

REFERENCES

(1) T. Morita, K. Ito, and T. Nose, *Arzneim.-Forsch. Drug Res.*, **32**, 1053 (1982).

(2) T. Morita, T. Kanazawa, K. Ito, and T. Nose, *Arzneim.-Forsch. Drug Res.*, **32**, 1047 (1982).

(3) T. Morita, T. Kanazawa, K. Ito, and T. Nose, *Arzneim.-Forsch. Drug Res.*, **32**, 1043 (1982).

(4) N. Taira, M. Kawada, and K. Satoh, *J. Cardiovasc. Pharmacol.*, **5**, 349 (1983).

(5) E. W. Thomas, *J. Chromatogr.*, **305**, 233 (1984).

(6) A. J. Sedman and J. G. Wagner, *J. Pharm. Sci.*, **65**, 1006 (1976).

(7) A. J. Sedman and J. G. Wagner, "AUTOAN—A Decision-Making Computer Program," Publication Distribution Service, Ann Arbor, Mich., 1974.

(8) C. M. Metzler, G. L. Elfring, and A. J. McEwen, *Biometrics*, **30**, 562 (1974).

(9) M. Gibaldi and D. Perrier, in "Pharmacokinetics," Marcel Dekker, New York, N.Y., 1975, pp. 69–80.

(10) H. G. Boxenbaum, S. Riegelman, R. M. Elashoff, *J. Pharmacokinet. Biopharm.*, **2**, 123 (1974).

(11) G. N. Rolinson and R. Sutherland, *Br. J. Pharmacol.*, **25**, 638 (1965).

(12) M. Gibaldi and D. Perrier, in "Pharmacokinetics," Marcel Dekker, New York, N.Y., 1975, p. 146.

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Inhibition of Human Polymorphonuclear Leukocyte Cell Responses by Ibuprofen

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Abstract □ The stimulation of cell swelling, cell aggregation, polymorphonuclear leukocyte locomotion, and lysosomal enzyme release in response to chemoattractant were all inhibited by ibuprofen, a nonsteroidal anti-inflammatory agent. The dosages needed to induce 50% inhibition (ID_{50}) were 5.9, 7.6, 60, and 95 μg/mL, respectively. Aside from the differences in ID_{50}, there was also a difference in the degree of maximum inhibition (I_{max}) of the complement C5a-stimulated responses observed, so that at achievable serum drug concentrations of 20–50 μg/mL, inhibition of 67–78% for cell swelling, 69–82% for cell aggregation, 20–35% for migration response, and 17–38% for lysosomal enzyme release were demonstrated. Also observed were a minor stimulatory effect on nitroblue tetrazolium reduction and an inhibitory effect on the ability to kill *Staphylococcus aureus*, but only at very high concentrations (~2 mg/mL).

Keyphrases □ Ibuprofen—inhibition of polymorphonuclear leukocyte cell responses □ Polymorphonuclear leukocytes—inhibition of response, ibuprofen □ Anti-inflammatory agents—ibuprofen, inhibition of polymorphonuclear leukocytes

Although the acute inflammatory reaction is usually the combined result of vascular, humoral, and cellular responses, there is considerable evidence that the contribution of the polymorphonuclear leukocyte[1] is indispensable (1). This is derived from studies showing inhibition of experimental inflammatory response in PMN-depleted animals, the presence of lysosomal enzymes in inflammatory exudates in humans, and the production of tissue damage in experimental animals by PMN lysosomal lysates. Moreover, one mechanism of action of the effects of a variety of anti-inflammatory drugs, which includes corticosteroids and colchicine, is their ability to inhibit the release of lysosomal enzymes from PMN. Consequently, it is important to determine whether nonsteroidal anti-inflammatory agents, in addition to their inhibitory activity on prostaglandin synthesis (2), also have direct inhibitory effects on PMN function. Although the inhibitory effect of these drugs on PMN migration has been shown previously (3–5), a systematic study to quantitate the effects on this and other PMN responses is lacking. Therefore, we studied and quantitated the effects of the anti-inflammatory drug ibuprofen on PMN responses.

EXPERIMENTAL SECTION

Preparation of Leukocytes and Serum—Blood for these studies was obtained from healthy male and female laboratory and hospital personnel. Polymorphonuclear cells were prepared from heparinized (50 U of preservative-free sodium heparin per mL of blood) whole blood by sedimenting erythrocytes with 6% hetastarch–whole blood at a ratio of 1:5. The leukocyte-rich plasma was washed once with a commercially obtained medium[2] at pH 7.4 and centrifuged at 500×*g*. The cell pellet was resuspended in the same medium, and the PMN concentration was adjusted to 5×10^6 PMNs/mL. This preparation was used for PMN locomotion and bactericidal assays. For leukocyte aggregation, swelling, and lysosomal enzyme release assays, the leukocyte pellet was prepared with Hank's balanced salt solution[1]. To lyse contaminating erythrocytes, the cells were resuspended in ice-cold 0.15 M NH_4Cl (pH 7.5) for 10 min, with five 2-min interval inversions, followed by another wash with HBSS. The final cell pellet was resuspended in HBSS to a cell concentration of 10^7 PMNs/mL. Serum was prepared from clotted whole blood.

Chemotactic Factor—The chemotactic factor used in PMN locomotion studies was derived from zymosan-activated serum prepared by incubation of 5 mg of zymosan with 1 mL of serum at 37°C for 30 min. A 3% solution of this material in the medium[2] was used in the lower compartment.

Partially purified complement C5a fragments were used as the chemotactic factor in leukocyte aggregation, swelling, and lysosomal enzyme release studies. C5a was derived by the activation of normal serum with zymosan in the presence of 1 M ε-aminocaproic acid[3] and purified by gel filtration[4] (6). Active fractions were identified by the lysosomal enzyme release assay, lyophilized, and then dialyzed against 0.1 M phosphate buffer (pH 7.4), and

[1] Polymorphonuclear leukocyte will be abbreviated throughout the text as PMN; Hank's balanced salt solution will be abbreviated as HBSS.

[2] Medium 199; Difco Laboratories, Detroit, Mich.

[3] Sigma Chemical Co., St. Louis, Mo.

[4] Sephadex G-100; Pharmacia Fine Chemicals, Piscataway, N.J.

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Table I—Effect of Ibuprofen on Various Chemotactic Factor (CF)-Stimulated Leukocyte Functions

Function	n	ID_{50}, μg/mL	Maximum Reversal of CF Effects (I_{max}, %)	Reversal of CF Effects at 20–50 μg/mL, %
PMN swelling	2	5.9	87	67–78
PMN aggregation	2	7.6	95	69–82
Chemotaxis	3	60	78	20–35
Lysosomal enzyme release				
Glucosaminidase	3	94	110	19–38
Elastase	3	95	100	17–34

stored at −90°C in small aliquots until use. Each preparation was standardized by dose–response studies to calculate maximum enzyme release (V_{max}) and the concentration of C5a needed to induce 50% of the maximum response (K_m) in PMNs. Three times this value ($K_m \times 3$) was then used in the experiments.

Ibuprofen Preparation—Ibuprofen[5] was prepared by dissolving 16.7 mg of the drug in 1 mL of 0.1 M Tris-HCl buffer, pH 8 (high pH values allowed solubilization of ibuprofen). The mixture was readjusted to pH 7.5, mixed thoroughly, and diluted to 8.35 mL with Tris buffer. The ibuprofen concentration at this point was 2 mg/mL and had an absorbance of 0.176 at 280 nm.

Leukocyte Locomotion Assay—Assays were performed in duplicate by using modified transparent acrylic Boyden chambers[6] and 13-mm diameter, 5-μm pore-size cellulose nitrate filters[7]. The modifications of the Boyden technique, as well as the methods used for calculating the locomotion index (LI) have been previously reported in detail (7).

To test the effect of ibuprofen on PMN locomotion, various concentrations of the drug were added to 5×10^5 PMNs in the upper compartment (total volume of 0.7 mL in the medium[2]). The chemotactic attractant, 3% zymosan-activated normal serum, was introduced into the lower compartment. The prepared chambers were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 90 min. After incubation, the filters were removed, fixed, stained, cleared, and mounted as described previously (7).

The effects of the drug on both the number of PMNs migrating and the locomotion index (distance migrated) were quantitated. To assess the total number of PMNs migrating into the filter, the number of cells was counted at a magnification of ×400. The counting was started at 20 μm below the cell monolayer (proximal filter surface) and continued at 10-μm intervals throughout the thickness of the filter. The locomotion index (LI_{20}), the average distance migrated by PMNs that migrated beyond the 20-μm level, was calculated as described previously (7). The chemotactic responses in the presence of ibuprofen were expressed as change from the control value measured in the absence of the drug.

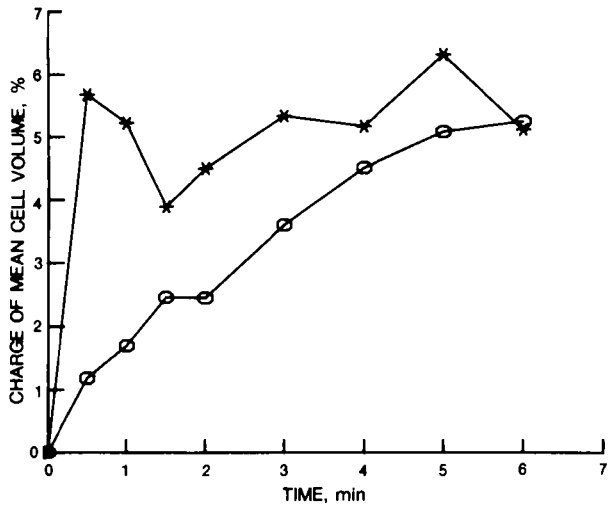

Figure 1—*Effect of ibuprofen on C5a-stimulated PMN swelling. For these experiments, 2×10^6 PMNs were exposed to 2.8 mg of ibuprofen per mL (total volume, 250 μL) for 2 min at 37°C, after which 100 μL of C5a solution ($K_m \times 3$) was added, and incubation was continued for an additional minute. An 80-μL aliquot was added to 20 mL of diluent, and the mean cell volume was determined at the times indicated. Key: (○) with ibuprofen; (✱) no ibuprofen.*

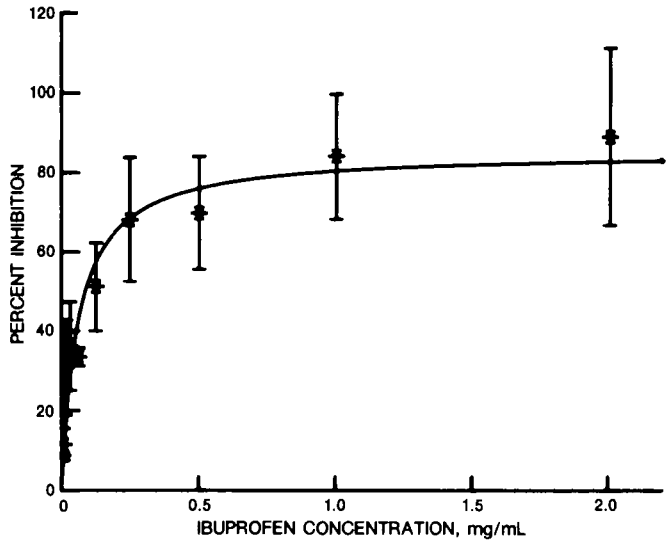

Figure 2—*Effect of ibuprofen on PMN locomotion (LI_{20}) toward activated normal serum as the chemoattractant. The results of three experiments are presented, with the bars representing* SD. *The ID_{50} was calculated to be 60 μg/mL, and the maximum inhibition was 78%.*

Lysosomal Enzyme Release Assay—Lysosomal enzyme release from PMNs was assessed by a method similar to that described by Fantone *et al.* (8). Reaction mixtures containing various concentrations of ibuprofen in the presence of cytochalasin B-treated PMNs (10^7 cells per mL) were preincubated at 37°C for 5 min. To these mixtures, 150 μL (K_m = 50 μL) of C5a was added. The mixtures were incubated at 37°C for 5 min and centrifuged at 200×*g* for 5 min at 4°C. The supernatant solutions were assayed for *N*-acetyl-β-D-glucosaminidase (8) and elastase (9).

Separation of C5a and Ibuprofen—In the study to determine whether ibuprofen has a direct effect on C5a, 2 mL of C5a was incubated with 2 mg of drug for 1 min at 37°C. The drug was then removed by using gel filtration columns[8]. An elution volume between 3.5 and 4.8 mL contained the highest concentration of C5a without the drug.

PMN Aggregation and Swelling—PMN aggregation and cell volume assays were performed by a technique described previously (6). We used a particle counter equipped with a size-distribution analyzer[9]. Reaction mixtures containing various concentrations of ibuprofen were incubated for 2 min at 37°C with 100 μL of PMNs (containing 2×10^6 PMNs). After incubation, 150 μL of C5a was added, and the mixtures were incubated for 60 s at 37°C. Aliquots of 80 μL from these reaction mixtures were added to 20 mL of isotonic solution[10] and immediately analyzed with a particle counter and size analyzer. The first determination of cell number and mean cell volume was obtained 15–30 s after contact of the PMNs with C5a. Subsequent readings were taken in the same vial which was intermittently shaken between readings. Cell number and mean cell volume were calculated for each drug concentration at all time intervals. A decrease in cell number was indicative of cell aggregation.

PMN Nitro Blue Tetrazolium Reduction and Bactericidal Assay—The methods of quantitating nitro blue tetrazolium reduction and bacterial killing by PMNs have been described previously (7). The only modification used for these assay procedures involved the preincubation of PMNs with various concentrations of ibuprofen for 30 min at 37°C.

Analysis of Results—To quantitate the inhibitory effects of ibuprofen, dose–response studies were performed to determine the degree of inhibition as a function of ibuprofen concentration. The percent inhibition (%I) was calculated from:

$$\%I = \left[\frac{R_D - R_{Neg}}{R_{C5a} - R_{Neg}} - 1\right]100$$

where the responses (R) are: R_D, in the presence of ibuprofen plus C5a fragments; R_{C5a}, in the presence of C5a fragments alone; R_{Neg}, in the presence

[5] Motrin; donated by the Upjohn Co., Kalamazoo, Mich.
[6] Ahlco Corp., Meriden, Conn.
[7] Sartorius Filters, Hayward, Calif.
[8] Column PD-10; Pharmacia Fine Chemicals.
[9] Model ZBI particle counter equipped with a model C-1000 Channelyzer; Coulter Electronics, Hialeah, Fla.
[10] Hematall; Fisher Scientific Co., Pittsburgh, Pa.

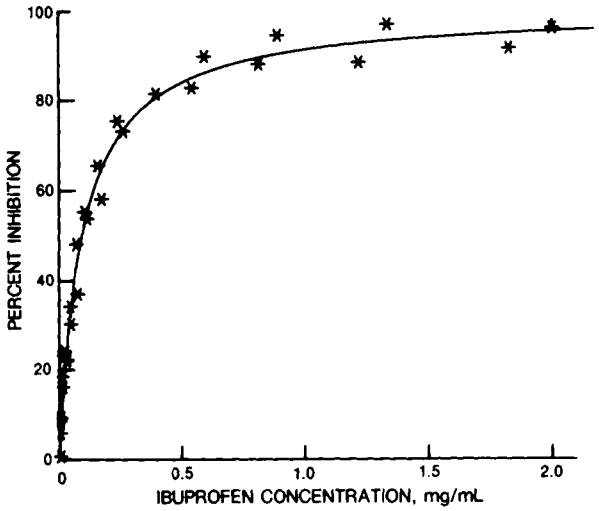

Figure 3—*Effect of ibuprofen on the C5a-stimulated release of the lysosomal enzyme elastase from PMNs. The results of three separate experiments are presented. The ID_{50} was calculated to be 95 μg/mL, and the maximum inhibition of enzyme release was 100%.*

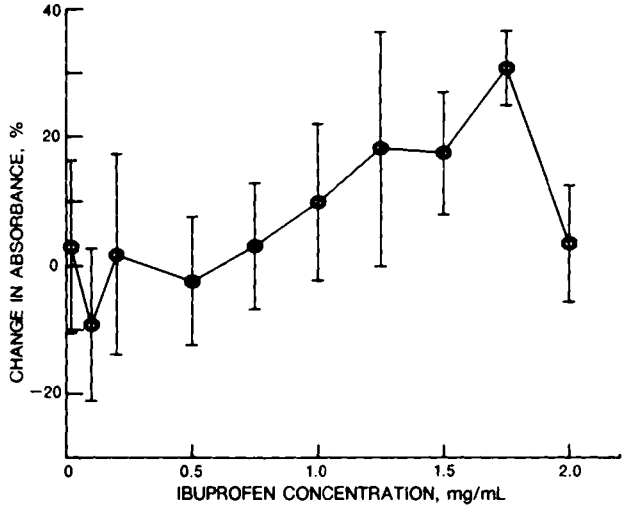

Figure 4—*Effect of ibuprofen on the reduction of nitroblue tetrazolium by PMNs. The percent change in absorbance at 550 nm as a function of ibuprofen concentration is presented for four separate experiments, with the bars representing the* SD. *Change in the positive direction indicates stimulation of nitro blue tetrazolium reduction.*

of neither. We found that the data conformed to a rectangular hyperbola of the form:

$$\%I = \frac{I_{max}\,[\text{ibuprofen}]}{ID_{50} + [\text{ibuprofen}]}$$

where I_{max} is the maximum reversal of chemoattractant effect expected when the ibuprofen concentration is extrapolated to infinite concentration, and ID_{50} is the concentration of ibuprofen that will produce $0.5 \times I_{max}$. The data were analyzed by nonlinear regression (10).

RESULTS

Ibuprofen inhibited all PMN responses to the chemoattractant. These included the locomotory chemotactic response (as measured by the micropore filter technique), lysosomal enzyme release, cell aggregation (as measured by a transient decrease in the number of PMN cells occurring within 30 s from contact with the chemoattractant), and cell swelling. These effects were directed against the cells and not against the chemoattractant, since treatment of C5a with ibuprofen and subsequent removal of the drug by gel filtration[8] did not impair subsequent C5a activity on release of the lysosomal enzyme elastase. The ID_{50} and I_{max} values with ibuprofen varied according to the response tested. A summary of these effects is shown in Table I. Cell swelling and cell aggregation had the lowest ID_{50} values, whereas these values were high for locomotory response and lysosomal enzyme release.

Of additional interest is the effect of ibuprofen on PMN cell swelling. Although it inhibited the rapid cell swelling that occurred 30 s after PMN contact with C5a, the drug itself produced a relatively slower swelling of cells (Fig. 1). The delayed swelling caused by ibuprofen, which eventually exceeded that produced by C5a, occurred with or without the presence of C5a. Six determinations of the effect of ibuprofen on PMN swelling and aggregation were made at an ibuprofen concentration of 1.75 mg/mL. We found that the inhibition of swelling was 90% (calculated value = 87%) and inhibition of aggregation was 96% (calculated value = 95%).

The locomotory index (distance migrated per cell) was inhibited, with an I_{max} value of 78% (Fig. 2), but the number of PMNs migrating did not diminish, except at very high concentrations (40% inhibition at 1.5–2.0 mg/mL) of the drug. Thus, the PMNs are affected in two distinctly different ways, depending on drug concentration. The reduced number of migrating PMNs at high concentrations of ibuprofen was not related to cell death or loss of viability since, in the trypan blue dye exclusion study, 98% of PMNs exposed to 2 mg of drug per mL excluded trypan blue dye, as compared with 94% of control cells. Also shown in the trypan blue dye study is that 55% of control cells had the triangular active morphology and were moving, whereas in the drug-treated PMNs only 12% assumed this active form. This is consistent with inhibition of locomotory response observed by the micropore filter assay.

Lysosomal enzyme release, particularly that of elastase, can be completely inhibited (I_{max} = 100%) by high concentrations of ibuprofen (Fig. 3). The reduced lysosomal enzyme activity observed in the presence of ibuprofen was due to reduced release of enzymes and not direct inhibition of the enzyme by the drug, since addition of ibuprofen after the enzyme release step of the assay, using control PMNs not previously treated with the drug, did not produce any change in the quantity of enzyme activity. There is a suggestion of individual variability in the ibuprofen response of PMNs. A donor with a low PMN locomotory response was found to have ID_{50} values of 191 μg/mL for glucosaminidase and 113 μg/mL for elastase release and I_{max} values of 111 and 113%.

Nitro blue tetrazolium reduction by PMNs was not affected, except at high concentrations of the drug. There was a linear increase ($p < 0.001$) in nitro blue tetrazolium reduction in the presence of 0.5–1.75 mg of the drug per mL and a sharp drop in activity thereafter (Fig. 4). Bactericidal activity against *Staphylococcus aureus* was also not affected by ibuprofen, except at a concentration of 2 mg/mL, at which a discernible inhibition was found (Fig. 5).

Perhaps of greater clinical relevance than the I_{max} values are the estimates of inhibition of the responses at drug concentrations of 20–50 μg/mL, since these are achievable in plasma. These estimates (Table I) indicate that PMN swelling and aggregation induced by C5a are most inhibited by these levels of ibuprofen. The chemotactic (locomotory) response to zymosan-activated normal serum is reduced by 20–35%, and the stimulation of lysosomal enzyme release by C5a is reduced by 17–38% (Table I).

DISCUSSION

The results of these studies indicate that ibuprofen may indeed exert its anti-inflammatory action on multiple sites. Besides its well-known effect on prostaglandin synthesis resulting from its potent inhibitory activity against the enzyme cyclo-oxygenase (11), it has easily demonstrable inhibitory effects on PMN responses to chemoattractant. By comparison, however, ibuprofen is much more active against cyclo-oxygenase than against PMN responses since the ID_{50} to platelet and endothelial cell prostaglandin synthesis was found to be 1 μg/mL [calculated from the data of Parks *et al.* (12)]. Relatively minimal effects on bactericidal function against *S. aureus* and on nitroblue tetrazolium reduction were also demonstrable at very high concentrations. Quantitative assessment of these effects indicates evidence of selectivity. For example, cell aggregation and swelling were inhibited by low concentrations of the drug that are easily achievable in serum with the usual oral doses, whereas lysosomal enzyme release and chemotactic (locomotory) responses required much higher concentrations. From these results, particularly with the effect of ibuprofen on chemotactic response, it is difficult to explain why the use of ibuprofen is not associated with a clinically obvious impairment of antimicrobial host defenses. It is likely that the combined effects on PMN responses and prostaglandin synthesis, but not separately, result in a predominantly anti-inflammatory effect. From these studies, it can be concluded that it is also likely that antimicrobial defenses may suffer at very high concentrations.

Because of its effect on PMN aggregation, it is possible that this drug and other nonsteroidal anti-inflammatory drugs may be useful in the prevention of disorders that result from vascular leukocytic stasis of intravascularly stimulated PMNs, such as adult respiratory distress syndrome related to open heart surgery, filtration leukapheresis, systemic infections, and hemodialysis

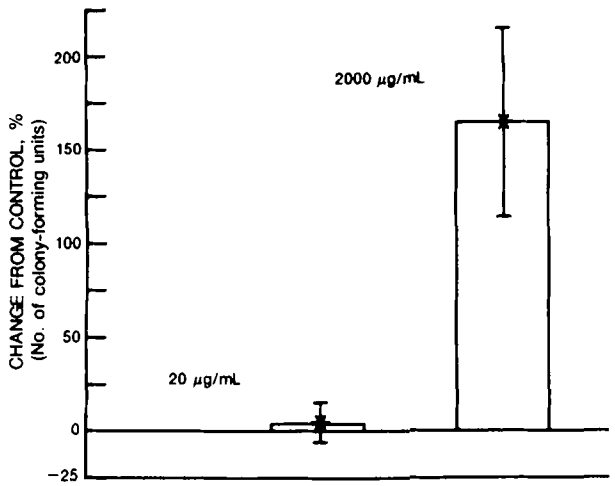

Figure 5—*Effect of ibuprofen on the bactericidal activity of PMNs against* S. aureus. *The percent change from the control is based on the number of colony-forming units remaining. Change in the positive direction indicates inhibition of the bactericidal activity of the cells.*

(13). Moreover, ibuprofen may be a useful agent to prevent the pulmonary complications of bleomycin, since new evidence indicates that this antineoplastic drug concentrates in PMNs and may be delivered at high concentrations to the lungs when conditions for the occurrence of pulmonary vascular leukocytic stasis are present (14).

The mechanism to explain the inhibitory effects of ibuprofen on PMNs is not known. Since aggregation, chemotaxis, and lysosomal enzyme release were inhibited almost to a similar degree by the drug, it is likely that the mechanism involves a pathway common to all three responses. These common pathways include the cell membranes and the cellular messengers, such as cell receptors, calcium, cyclic neucleotides, and arachidonic acid metabolites, particularly of the lipoxygenase pathway. Although the lysosomal membrane was "stabilized" by ibuprofen and other nonsteriodal anti-inflammatory drugs, as reported by this study and others (15), there is yet little direct evidence of cell membrane inhibitory effects by these drugs. Recent evidence points to a regulatory influence of prostaglandins on cyclic neucleotides and calcium (16). Although the effects of specific postaglandins on cyclic nucleotides may be known, it is not possible to predict what effects the inhibition of prostaglandin synthesis would have. This is because prostaglandin effects are concentration-dependent (a low concentration of PGE lowers, while a high cocentration raises, cAMP cellular levels), and also dependent upon the specific compound (*i.e.*, PGF_{2a} increases cGMP; PGE and PGD increases cAMP) (17, 18). Moreover, the effects of the early unstable metabolites (endoperoxides and thromboxane) differ from that of the stable prostaglandins (19).

REFERENCES

(1) I. M. Goldstein, in "Mediators of Inflammation," G. Weissmann, Ed., Plenum, New York, N.Y., 1974, p. 51.

(2) J. R. Vane, *Nature (London) New Biol.*, **231,** 232 (1971).

(3) E. Magluilo, R. Genzi-Cipelli, A. Lago, M. Azzini, E. Mogin, and S. Gallico, *Int. J. Clin. Pharmacol.*, **15,** 417 (1977).

(4) S. Spisani, G. Vanzini, and S. Tramiello, *Experientia*, **35,** 803 (1979).

(5) G. A. Higgs, K. E. Eakins, K. G. Mugridge, S. Moncada, and J. R. Vane, *Eur. J. Pharmacol.*, **66,** 81 (1980).

(6) J. T. O'Flaherty, D. L. Kreutzer, and P. A. Ward, *J. Immunol.*, **119,** 232 (1977).

(7) E. G. Maderazo and P. A. Ward, in "Todd-Sanford-Davidsohn Clinical Diagnosis by Laboratory Methods," Vol. 2, 16th ed., J. B. Henry, Ed., W. B. Saunders Co., Philadelphia, Pa., 1979, p. 1335.

(8) J. Fantone, R. M. Senior, D. L. Kreutzer, M. Jones, and P. A. Ward, *J. Lab. Clin. Med.*, **93,** 17 (1979).

(9) M. J. Castillo, K. Nakajima, M. Zimmerman, and J. C. Powers, *Anal. Biochem.* **99,** 53, (1979).

(10) C. L. Woronick, J. Malnick, and E. G. Maderazo, *J. Lab. Clin. Med.*, **98,** 58 (1981).

(11) T. G. Kantor, *Ann. Intern. Med.*, **91,** 887 (1979).

(12) W. M. Parks, J. C. Hoak, and R. L. Czervoike, *J. Pharmacol. Exp. Ther.*, **219,** 415, (1981).

(13) D. E. Hammerschmidt, L. J. Weaver, L. D. Hudson, P. R. Craddock, and H. S. Jacob, *Lancet*, **i,** 947, (1980).

(14) J. T. Noble, K. Ramberg-Laskaris, and M. S. Klempner, Program Abstracts of the 22nd Interscience Conference on Antimicrobial Agents and Chemotherapy (abstract No. 41), 1982, p. 72.

(15) R. J. Smith, *J. Pharmacol. Exp. Ther.*, **207,** 618 (1978).

(16) D. F. Horrobin, "Prostaglandins. Physiology, Pharmacology and Clinical Significance," Eden Press, Montreal, Canada, 1978, p. 151.

(17) H. R. Hill, in "Leukocyte Chemotaxis. Methods, Physiology and Clinical Implications," J. I. Gallin and P. G. Quie, Eds., Raven, New York, N.Y., 1978, p. 179.

(18) C. J. Dunn, D. A. Willoughby, J. P. Giroud, and S. Yamamoto, *Biomedicine*, **24,** 214, (1976).

(19) G. Weissmann, "Prostaglandins in Acute Inflammation. Current Concepts," monograph published by Upjohn Co., Kalamazoo, Mich., 1980.

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