Agents and Actions, vol. 26, 3/4 (1989)

0065-4299/89/040335-07 $ 1.50+0.20/0

Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents

L.M. De Young, J.B. Kheifets, S.J. Ballaron and J.M. Young

Department of Inflammation Biology, Syntex Research, 3401 Hillview Avenue, Palo Alto, CA 94304, USA

Abstract

The temporal patterns of edema and accumulation of the PMN marker enzyme, myeloperoxidase (MPO), were examined following application of tetradecanoylphorbol acetate (TPA) to mouse ears. After application of 2.5 μg TPA, edema peaked at 6 hr, while MPO activity peaked at 24 hr. Pharmacological agents with defined mechanisms of action, delivered orally or topically, were assessed for effects on these responses. For oral administration, compounds were delivered 1 hr before and 6 hr after TPA and for topical administration compounds were delivered at 15 min and 6 hr after TPA. Topical and oral corticosteroids inhibited both edema and MPO accumulation. Cyclooxygenase and lipoxygenase inhibitors were very effective against MPO accumulation but were either inactive or moderately active vs edema. Anti-histamine/anti-serotonin agents had little effect on edema, but could inhibit or exacerbate MPO accumulation depending on dose and route of administration. Topically applied histamine itself did not effect TPA-induced edema, but markedly suppressed MPO accumulation. Acetone, the vehicle, when topically applied between 0.5 and 2 hr after TPA inhibited MPO accumulation by 60–80%, but had little effect on edema. Acetone applied before 0.5 hr or after 2 hr had no effect on either parameter. These results indicate that in the TPA-induced ear inflammation model the MPO response at 24 hr may be a useful additional indicator of drug activity.

Introduction

Tetradecanoyl phorbol acetate (TPA)-induced mouse ear inflammation has been used to study both the mechanism and inhibition of inflammation [1, 2]. In these studies, measurement of edema was the primary endpoint, although cell infiltration may be a more relevant indicator of tissue destruction. In other models of dermal inflammation, myeloperoxidase (MPO), a PMN marker enzyme, has been used to provide a quantitative measure of PMN infiltration in inflamed skin [3–5]. In the present study, we have used the MPO technique to help define the cellular response to TPA and to compare this cellular response to the kinetics of edema. In addition, we have examined the possibility that pharmacologic agents may have differential effects on these responses to TPA.

Materials and methods

Tested drugs and their sources were as follows: Dexamethasone, cimetidine, indomethacin, ibuprofen, histamine, cyproheptadine HCL, chlorpheniramine maleate (Sigma); clobetasol propionate (Glaxo); fluocinolone acetonide, lonapalene, ketorolac, and naproxen (Syntex); dapsone, leva-

misole, and diphenyldisulfide (Aldrich); auranofin (SKF); BW755C HCL (Wellcome); Na zomepirac (McNeil).

TPA treatment

Female Sim: (SW)fBR mice, 7–8 weeks old, were randomly assigned to groups of 6. The right ear of each mouse received 2.5 µg TPA (CCR Inc.) in 20 µl acetone (10 µl to each side of the ear). Control animals received acetone only.

Edema measurements

To study the inflammatory effects of TPA, 6 mm diameter tissue plugs were taken from the right ears of CO_2-euthanized mice at the specificed times and weighed. For determining inhibition of edema by drugs, ear thickness was measured with a dial thickness gauge at 6 hr post TPA so that the 6 hr edema and 24 hr MPO measurements could be determined in the same group of animals. In pilot studies ear thickness and wet weight of tissue plugs gave comparable results.

Microscopy

For histological evaluation, formalin-fixed tissue was embedded in paraffin, sectioned and then stained with the May-Grunwald Giemsa method. For the histochemical study of MPO positive cells, fresh tissue was frozen in O.C.T. compound (Miles Laboratories), sectioned and stained using Sigma Diagnostic kit # 391 A.

Myeloperoxidase assay

The technique of Suzuki et al. [6] was modified to utilize a microtiter plate reader. A 6 mm ear tissue punch was placed in 0.75 ml of 80 m*M* sodium phosphate buffer, pH 5.4, containing 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma). The sample was then quick frozen on Dry Ice and stored at −20 °C until assayed. For assay, samples were thawed at room temperature and homogenized for 45 sec at 0°C in a motor driven glass/glass homogenizer. Homogenate was decanted into a microfuge tube, and the vessel was washed with a second 0.75 ml aliquot of HTAB in buffer. The wash was added to the tube and the 1.5 ml sample was centrifuged at 12000 × *g* at 4 °C for 15 min. Triplicate 30 µl samples of the resulting supernatant were added to 96 well microtiter plates. For assay, 200 µl of a mixture containing 100 µl phosphate buffered saline, 85 µl 0.22 *M* sodium phosphate buffer, pH 5.4, and 15 µl of 0.017% hydrogen peroxide were added to the wells. The reaction was begun by the addition of 20 µl of 18.4 m*M* tetramethylbenzidine HCl (Sigma) in 8% aqueous dimethylformamide. Plates were incubated at 37 °C for 3 min and then placed on ice where the reaction was stopped by addition to each well of 30 µl of 1.46 *M* sodium acetate, pH 3.0. Enzyme activity was determined colorimetrically using a Dynatech plate reader set to measure absorbance at 630 nm. Activity is expressed as units of OD/min/ml enzyme supernatant.

Drug testing

For oral administration, drugs were suspended in a vehicle containing 0.9% NaCl, 0.5% Na carboxymethylcellulose, 0.4% polysorbate 80, 0.9% benzylalcohol and 97.3% H_2O (CMC). Volumes of 0.01 ml/g body weight were administered by gavage at 1 hr before and 6 hr after TPA application. Control animals were given CMC vehicle only and topically treated with either TPA (stimulated control) or acetone (baseline control).

For topical application, drugs were dissolved in acetone and applied to the right ear in a volume of 20 µl (10 µl to each surface) 15 minutes and 6 hr after TPA application. Control animals received either TPA once and acetone twice (stimulated control) or acetone three times (baseline control). For both topical and oral drug testing, edema was determined at 6 hr by measuring ear thickness just prior to the second administration of drug. Thus, only the first dose of drug could effect edema. The second (6 hr) drug treatment was included to optimize the opportunity for a test compound to act on the later (24) hr peak of MPO activity. The percent change for both edema and MPO accumulation was calculated as follows: [(TPA and drug treated − baseline control/stimulated control − baseline control) −1] × 100.

Statistics

Data were analyzed using a one-way analysis of the variance followed by a Newman-Keuls mul-

tiple range test. The p-values given in tables and figures refer to the differences in original unit mean value. These values and their standard deviations were normalized to percent control for presentation. TPA-challenged animals receiving vehicle only represent 100% stimulation, and the acetone challenged animals represent 0% stimulation.

Results

Edema and MPO accumulation after TPA

Following topical application of TPA the increases in edema and MPO had distinctly different kinetics (Fig. 1). Edema peaked at 6 hr and then rapidly waned. In contrast, MPO activity peaked at 24 hr and then slowly decreased.

Acetone vehicle effect

Previous studies have demonstrated that acetone inhibits mouse ear edema when applied at certain times after TPA [1]. Since we wished to use acetone as topical vehicle, we undertook studies to confirm this phenomenon. As seen in Figure 2, acetone exerted a mild (20%) inhibition of edema applied at either 30 min or 1 hr after TPA, while treatment at other times had no effect. However, acetone inhibited the 24 hr peak of MPO activity from 60–80%, when applied at 0.5, 1 or 2 hr after TPA. Therefore, to avoid these effects of the vehicle acetone, topical drugs were delivered at 15 min and 6 hr after TPA.

Pharmacological Modulation of TPA-induced edema and MPO accumulation

The marked temporal separation of the maximum edema and MPO responses suggested that they may be differentially modulated by pharmacologic agents. Tables 1 and 2 show the effects of oral and topical drugs respectively on TPA-induced mouse ear inflammation. The cyclooxygenase inhibitors, indomethacin, ketorolac, naproxen, and zomepirac administered orally were effective inhibitors of MPO accumulation but poor inhibitors of edema (Table 1). Oral ibuprofen supressed MPO activity without any effect on edema. When topically applied, ibuprofen, indomethacin, naproxen, and ketorolac also preferentially inhibited MPO accumulation (Table 2).

Topical application of the anti-psoriatic drugs, anthralin and lonapalene also resulted in a dramatic suppression of MPO activity. Anthralin at

Table 1
The effect of orally delivered drugs on edema and myeloperoxidase activity in the TPA-treated mouse ear.

Drug	Dose mg/kg	Percent change [1]	
		Edema [2]	MPO [3]
Antiinflammatories and Antirheumatics			
Aspirin	200	− 3	−11
Auranofin	10	−24*	−40**
Dexamethasone	0.10	−31**	−40**
	0.50	−57**	−96**
Levamisole	100	0	−11
Ibuprofen	100	0	−61**
	200	1	−69**
Indomethacin	1	− 7	−33*
	5	−10	−34**
	10	−15*	−66**
Ketorolac	1	− 5	−12
	5	−10	−22*
	10	−21*	−55**
Naproxen	1	− 6	− 4
	5	−10	−35**
	10	−15*	−45**
Phenidone	100	−18	−46**
Sulfasalazine	200	−13	0
Zomepirac	50	−30*	−83**
Mediator Antagonists			
Chlorpheniramine	1	− 2	−14*
	5	− 8	−41**
	50	13	78**
Cimetidine	0.5	−11	− 8
	5	−10	18
	50	− 2	102**
Cyproheptadine	5	− 2	−12
	25	− 2	0
	50	5	−57**
Promethazine	5	−17	−48**
	50	0	−36**

[1] Negative and positive values indicate inhibition and stimulation, respectively.
[2] Average value of stimulated and baseline controls for all experiments = 0.46 mm and 0.21 mm, respectively.
[3] Average value of stimulated and baseline control for all experiments = 47.52 OD/min/ml and 0.53 OD/min/ml, respectively.
* $p < 0.05$. ** $p < 0.01$.

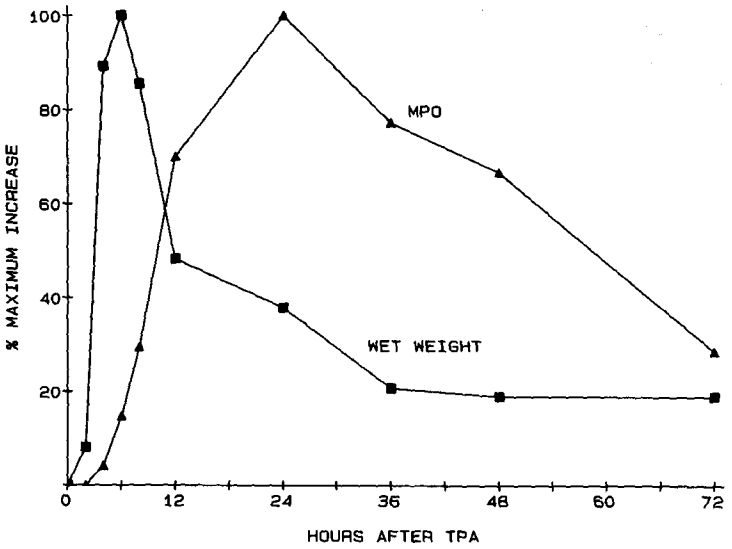

Figure 1
Measurement of edema and MPO accumulation after TPA treatment. Mean ± SD maximal (100%) increase for wet weight and MPO accumulation respectively were 11.1 ± 2.1 mg and 33.34 ± 2.9 OD/min/ml ($n = 6$ right ears).

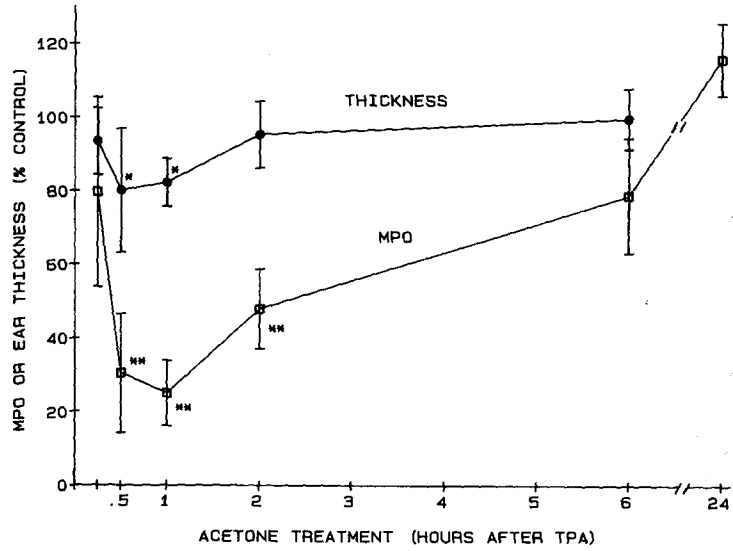

Figure 2
The effect of acetone treatment on TPA-induced mouse ear edema and MPO accumulation. Acetone was applied at the times indicated after TPA. Ear thickness and MPO activity were determined at 6 and 24 hr respectively after TPA. Control (100%) mean ± SD values for ear thickness and MPO respectively were 0.46 ± 1.9 mm and 32.26 ± 7.18 OD/min/ml ($n = 6$ right ears). * $p < 0.05$ vs control, ** $p < 0.01$ vs control.

Table 2
The effect of topically delivered drugs on edema and myeloperoxidase activity in the TPA-treated mouse ear. ($n = 1-3$ experiments).

Drug	Dose mg/ear	Percent change [1]	
		Edema [2]	MPO [3]
Antiinflammatories			
Ibuprofen	0.1	29**	13
	1.0	−44**	− 85**
Indomethacin	0.1	−19*	− 77**
	1.0	−48**	−100**
Ketorolac	0.1	6	− 55**
	1.0	−46**	−100**
Naproxen	0.1	− 6	− 87**
	1.0	−38**	− 93**
Phenidone	0.1	−34**	− 98**
	1.0	−54**	−100**
Mediator Antagonists			
Chlorpheniramine	0.1	−16	− 60**
	1.0	−24*	− 63**
Cimetidine	0.1	− 2	− 60**
	1.0	− 3	− 84**
Miscellaneous			
Histamine [4]	0.2	− 6	− 34**
	2.0	0	− 76**
Anthralin	0.1	−11	− 68**
Lonapalene	0.1	− 2	− 30**
	0.5	−13	− 67**
	1.0	−31**	− 92**
	2.0	−34**	− 95**

[1] Negative and positive values indicate inhibition and stimulation, respectively.
[2] Average value of stimulated and baseline controls for all experiments = 0.45 mm and 0.21 mm, respectively.
[3] Average value of stimulated and baseline control for all experiments = 33.12 OD/min/ml and 1.02 OD/min/ml, respectively.
[4] Delivered in 100% ETOH.
* $p < 0.05$. ** $p < 0.01$.

0.1 mg/ear did not inhibit edema and was not tested at higher doses due to its known pro-inflammatory activity [2]. Lonapalene, a specific 5-LO inhibitor [7] inhibited MPO activity at much lower doses than those required for significant inhibition of edema (Table 2).

At all doses tested, oral chlorpheniramine, cimetidine, cyproheptadine and promethazine were inactive against edema. However, at a dose of 5 mg/kg chlorpheniramine inhibited MPO by 40%, while at a dose of 50 mg/kg MPO activity was increased 78%. Similarly, cimetidine did not cause significant changes at doses below 5 mg/kg but at 50 mg/kg it doubled TPA-induced MPO activity (Table 1).

When topically tested, only chlorpheniramine exhibited a weak inhibition of edema, although both chlorpheniramine and cimetidine significantly suppressed MPO activity (Table 2). Topically applied histamine was itself an inhibitor of MPO activity (Table 2).

Since topical corticosteroids are the only compounds with well established dermal anti-inflammatory potencies, we tested a medium (dexamethasone), high (fluocinolone acetonide), and super (clobetasol propionate) potency steroid in our system. These compounds exhibited the expected rank order for both edema and MPO inhibition (Fig. 3).

All active drugs were tested at 10^{-4} *M* for direct effects on MPO in supernatants obtained from TPA-only treated ears. Drugs were added in dimethylformamide directly to the assay mix. The concentration of drug was calculated to be much greater than that which could possibly be carried over into the assay after *in vivo* oral or topical treatment. Under these conditions, auranofin, zomepirac and indomethacin inhibited MPO activity by 30–40%, while all other drugs with the exception of anthralin and phenidone inhibited by 0–20%. Both anthralin and phenidone gave 100% inhibition of the MPO reaction. No tested drug exhibited a significant direct stimulation of activity. Thus only for anthralin and phenidone could a direct inhibition of MPO plausibly explain the results observed after *in vivo* treatment.

Light Microscopy

Biochemical determination of MPO indicated the presence of many more PMNs at 24 hr than at 6 hr after TPA. This agreed with our histological and histochemical studies which demonstrated many more PMN's and MPO positive cells at 24 hr than at 6 hr. Similarly, in tissue sections taken from TPA-treated ears that were topically treated with 1.0 mg indomethacin 15 min and 6 hr after TPA (a regimen that inhibited MPO activity by > 90%), the increase in MPO positive cells observed at 24 hr was virtually abolished and the number of

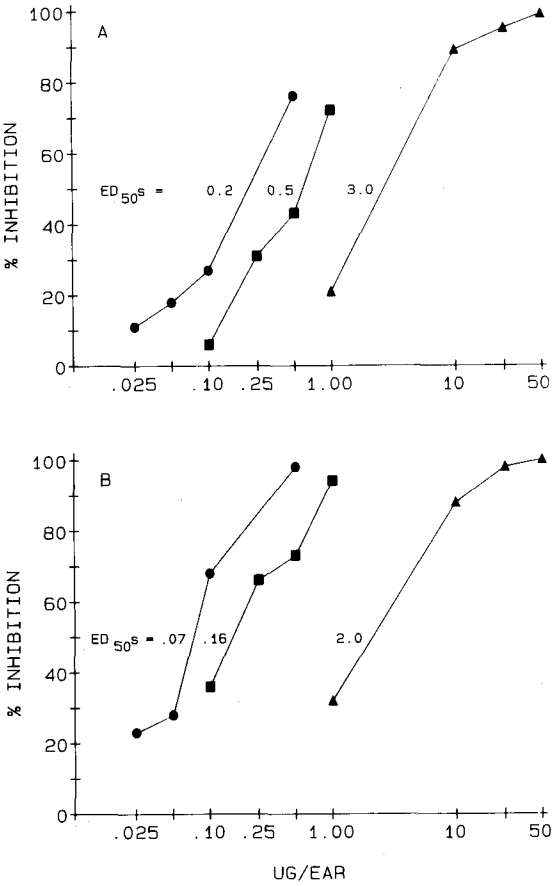

Figure 3
The relative inhibitory effects of topical steroids against TPA-induced edema (ear thickness) (A) and MPO accumulation (B). (●) clobetasol propionate, (■) fluocinolone acetonide, (▲) dexamethasone. ED_{50} = dose that effects a 50% inhibition.

PMNs appeared markedly diminished. However, there did not appear to be a concomitant decrease in the number of infiltrating mononuclear cells (Results not shown).

Discussion

Extending the TPA-induced mouse ear inflammation model to include MPO as well as edema measurements as an endpoint led to several new observations. Foremost, is the marked separation of responses with edema peaking at 6 hr and MPO activity peaking at 24 hr. A similar, but less dramatic separation of edema and MPO activity has been reported to occur during reverse passive Arthus reactions in rat skin [4, 8].

Our results clearly show that the inability of a drug to inhibit edema may not predict a lack of effect on cell infiltration. Indeed, we found that many active compounds were more effective against MPO accumulation than against edema. Thus, cycloxygenase and lipoxygenase inhibitors and anti-histamine/anti-serotonin agents can profoundly alter MPO accumulation while having either no effect or a moderate inhibitory effect on edema. The ability of cyclooxygenase inhibitors to suppress TPA-induced cell infiltration is consistent with other studies, showing that they can inhibit the migration of neutrophils into inflammatory sites [4, 9, 10]. Interestingly, our histological investigations as well as those of Bailey and Sturm [4], suggest that cyclooxygenase inhibitors supress PMN infiltration without affecting the influx of monocytes. In our study, monocytes would not be expected to significantly contribute to total MPO activity. In ears treated only with TPA, monocytes comprised a minor fraction of the total cell infiltrate which was predominated by PMN's, and in the rat, on a per cell basis, monocytes have only about 1% of the MPO activity found in PMN's [3].

Phorbol esters are known to induce degranulation of mast cells [11] and cause histamine accumulation and vascular leakage in subcutaneous air pouches [12]. In the air pouch study [12], histamine and serotonin antagonists suppressed edema at 30 min but not at the peak response 4 hr post phorbol treatment. Similarly, in our study these antagonists did not inhibit edema at 6 hr, but could either inhibit or stimulate TPA-induced MPO accumulation depending on the dose used and route of administration. Thus, at low doses, cimetidine was inactive orally but at the high dose exacerbated TPA-stimulated MPO. In contrast, topically applied cimetidine was a potent inhibitor of MPO accumulation.

Oral chlorpheniramine exhibited a bi-modal response with low doses inhibiting and high doses stimulating MPO accumulation. However, when applied topically, chlorpheniramine, like cime-

tidine, was a potent inhibitor of MPO accumulation. Perhaps the explanation for these seemingly contradictory results, involves the effects of histamine itself. We found that topically applied histamine had no effect on edema while significantly suppressing MPO accumulation. Antihistamines can exhibit either antagonist or agonist effects depending on dose [13], and are thus theoretically capable of either potentiating or suppressing the ability of endogenous histamine to inhibit myeloperoxidase accumulation.

It is noteworthy that of all the compounds tested only corticosteroids were potent inhibitors of both edema and MPO accumulation. Furthermore, topical steroids showed relative potencies in close agreement with their well established experimental and clinical rank order.

Carlson et al. [1] reported that acetone applied 15 min after TPA caused a 10–30% reduction in ear thickness at 4 hr post TPA. We measured ear thickness at 6 hr post TPA and found no significant effect on edema when acetone was delivered at 15 min and only a 20% suppression when delivered at 0.5 or 1.0 hr. However, acetone applied at 0.5 to 2 hr after TPA elicited a dramatic 60–80% inhibition of MPO accumulation measured at 24 hr. Acetone applied at 15 min after TPA exerted an insignificant inhibition of MPO, suggesting the possibility that an acetone-labile mediator is present from 30 min to 2 hr after TPA.

Besides the latter unusual acetone effects, we feel our most significant finding is that monitoring drug effects on MPO accumulation identifies anti-inflammatory compounds which ordinarily would be considered inactive or mildly active based on their activity against TPA-induced edema. Clearly, with the addition of MPO as an endpoint more compounds are positive in the TPA mouse ear test. For any novel compound showing activity against MPO accumulation, the potential therapeutic significance must be further assessed in disease specific models or clinical tests.

References

[1] R. P. Carlson, L. O'Neill-Davis, J. Chang and A. J. Lewis, *Modulation of mouse ear edema by cyclooxygenase and lipoxygenase inhibitors and other pharmocological agents.* Agents and Actions *17,* 197–204 (1985).

[2] J. M. Young, B. M. Wagner and D. A. Spires, *Tachyphylaxis in 12-O-tetradecanoylphorbolacetate- and arachidonic acid-induced ear edema.* J. Invest. Dermatol. *80,* 48–52 (1983).

[3] P. P. Bradley, D. A. Priebat, R. D. Christensen and G. Rothstein, *Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker.* J. Invest. Dermatol. *78,* 206–209 (1982).

[4] P. J. Bailey and A. Sturm, *Immune complexes and inflammation: The reverse passive arthus reaction in the rat.* Biochem. Pharmacol. *32,* 475–481 (1983).

[5] L. M. De Young, D. A. Spires, J. Kheifets and T. G. Terrell, *Biology and pharmacology of recombinant human interleukin-1B-induced rat ear inflammation.* Agents and Actions *21,* 325–327 (1987).

[6] K. Suzuki, H. Ota, S. Sasagawa, T. Sakatani and T. Fukikura, *Assay method for myeloperoxidase in polymorphonuclear leucocytes.* Anal. Biochem. *132,* 345–352 (1983).

[7] D. V. Krishnamurthy, M. Kruseman-Aretz, S. Rouhafza-Fard, C. J. Bedord, J. M. Young, G. Jones and M. Venuti, *Selective inhibition of arachidonic acid 5-lipoxygenase by a novel antipsoriatic agent, 6-chloro-1,4-diacetoxy-2,3-dimethoxy-naphthalene (RS-43179).* Fed. Proc. *44,* 886 (1985).

[8] D. S. Fletcher, D. Osinga and R. J. Bonney, *Role of polymorphonuclear leucocytes in connective tissue breakdwon during the reverse passive arthus reaction.* Biochem. Pharmacol. *35,* 2602–2606 (1986).

[9] M. S. Koh, L. Parente and D. A. Willoughby, *Immunological and non-immunological pleural inflammation: The effects of anti-inflammatory and anti-rheumatic drugs.* Eur. J. Rheum. Inflammation *1,* 286–290 (1979).

[10] A. C. Issekutz, M. Szpejda and M. Ripley, *Effects of lipoxygenase and cyclooxygenase inhibitors on neutrophil margination, emigration, and related inflammatory responses.* In *Advances in Inflammation Research,* vol. 11. (Ed. I. Otterness et al.) pp. 31–45, Raven Press, New York 1986.

[11] M. E. Cantwell and J. C. Foreman, *Phorbol esters induce a slow, non-cytotoxic release of histamine from rat peritoneal mast cells.* Agents and Actions *20,* 165–168 (1987).

[12] K. Ohuchi, M. Watanabe, C. Takahashi, Y. Hayashi, N. Hirasawa, S. Tsurufuji, H. Fujiki and T. Sugimura, *Analysis of tumor-promoter-induced inflammation in rats: participation of histamine and prostaglandin* E_2. Biochim. Biophys. Acta *925,* 156–163 (1987).

[13] L. G. Garland, A. F. Green and H. F. Hodson, *Inhibitors of the release of anaphylactic mediators.* In Anti-Inflammatory Drugs. (Ed. J. R. Vane and S. H. Ferreira) p. 515, Springer, New York 1979.