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IMMUNE COMPLEXES AND INFLAMMATION

A STUDY OF THE ACTIVITY OF ANTI-INFLAMMATORY DRUGS IN THE REVERSE PASSIVE ARTHUS REACTION IN THE RAT

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Abstract—The reverse passive Arthus reaction in rat skin was quantitated by using increase in wet weight as a measure of edema and extractable myeloperoxidase as a measure of the intensity of polymorphonuclear leukocyte (PMN) infiltration. Treatment of the animal with dexamethasone prior to challenge with antigen/antibody resulted in an inhibition of both edema and the infiltration of inflammatory cells. In contrast, the non-steroidal anti-inflammatory drugs inhibited the intensity of cell infiltration (PMNs but not mononuclear cells) without affecting edema. The results are discussed in the light of the effects of these drugs on arachidonic acid oxidation.

The Arthus reaction in the skin is an example of an inflammatory response caused by deposition of immune complexes. Tissue damage occurs due to the activation of complement components, the infiltration by polymorphonuclear leukocytes (PMNs)†, and the release of lysosomal enzymes, together with an increase in vascular permeability resulting from damage to the blood vessel wall [1]. The pathology of rheumatoid arthritis is also associated with circulating and intraarticular immune complexes which seem to figure prominently in the pathogenesis of the disease [2]. A study of the inflammatory response and the mechanism of tissue damage occurring in the Arthus reaction would, therefore, seem to have some relevance to that which occurs in rheumatoid arthritis.

The experiments reported here examine the effect of standard anti-inflammatory drugs on two of the major components of the Arthus reaction: (a) edema formation as measured by the increase in wet weight at the challenge site, and (b) cell infiltration (in this case PMNs) as measured by the amount of myeloperoxidase (MPO, EC 1.11.1.7) present at high specific activity in PMNs [3] extractable from the reaction site. The data show that both steroid and non-steroidal anti-inflammatory drugs affect the accumulation of PMNs at the site of the challenge. Non-steroidal agents, at anti-inflammatory dose levels in contrast to the steroid, do not inhibit edema formation but show a consistent inhibition of PMN accumulation. The pharmacological effects of these compounds are discussed in the light of their known effects on arachidonic acid metabolism.

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† Abbreviations: PMN, polymorphonuclear leukocyte; PBS, phosphate-buffered saline; RPA, reverse passive Arthus reaction; MPO, myeloperoxidase; and HTAB, hexadecyltrimethylammonium bromide.

MATERIALS AND METHODS

The Arthus reaction was assayed in non-sensitized animals using the reverse passive procedure by administering the antibody locally in the skin and injecting the antigen intravenously in the tail (referred to as the RPA).

Male Sprague–Dawley rats weighing 130–160 g (Taconic Farms, Germantown, NY) were housed in wire-bottomed Metronic cages and fed and watered *ad lib*. Twenty-four hours before the initiation of the RPA, the rats were sorted into groups of six animals, placed in boxes, and fasted overnight. Fasted rats showed a more reproducible response to drug treatment, and fasting did not measurably affect the Arthus response. Compounds were administered perorally as a solution or suspension in 0.25% agar in water. Uniformity of the drug suspension was ensured by homogenization in a Virtis 23 homogenizer for 5 min. The reverse passive Arthus reaction was induced using bovine serum albumin as antigen injected into the tail vein and rabbit anti-bovine serum albumin (R-ABSA) into the skin site. The injection of undiluted R-ABSA into the skin caused a non-specific inflammatory response in the absence of antigen. The R-ABSA was, therefore, diluted 1:4 with phosphate-buffered saline (PBS), which in the skin was found to produce a minimal inflammatory response in the absence of intravenously injected antigen, but which resulted in a good edematous and cellular response when antigen was injected into the tail vein. One hour after dosing with vehicle or drug, animals were lightly anesthetized with Metaphane, and the hair was shaved from the mid-dorsal region with electric clippers. Immediately after shaving, each animal was injected intradermally with 40 μl PBS at a site on the left side of the mid-dorsal line. Forty microliters of rabbit anti-bovine serum albumin (5.0 mg/ml antibody protein, Cappel Laboratories, Lot No. 13528), diluted

1:4 with PBS, was injected intradermally at a site on the right side of the dorsal midline, each skin site receiving 0.05 mg antibody protein. Immediately following the intradermal challenge, each rat received 0.5 ml PBS containing 1.0 mg bovine serum albumin (Miles Laboratories, Lot No. 50) injected in the tail vein).

Four hours after intradermal challenge, each group of animals was killed by asphyxiation in CO_2. The full thickness skin was removed from the back of each animal and a disc 8 mm in diameter was punched out with a metal punch. The skin samples from the PBS and antibody-injected site were placed on filter paper moistened with PBS. The wet weight of each skin site was determined as soon as possible after removal from the animal. The edema caused by the RPA is measured as the difference (expressed in mg) between the wet weight of the antibody-injected site and the PBS-injected site. That wet weight measurements are a true measure of extravasation of fluid from the blood vessels into the tissue was confirmed using [^{125}I]BSA. Leakage of the iodinated protein into the skin site was found to parallel the increase in wet weight. Also the inhibition of [^{125}I]BSA leakage and the wet weight determination obtained with dexamethasone treatment were of the same order, suggesting that the two measurements were indeed a true reflection of edema formation.

The main infiltrating cell in the RPA is the PMN which, in contrast to the macrophage and other local connective tissue cells, contains MPO at high specific activity [3, 4]. The amount of MPO in rat macrophages is less than 10% of that present in PMNs (unpublished observations). The amount of MPO in the skin sample is, therefore, a reflection of the number of infiltrating PMNs. MPO was extracted by placing each skin site in 10 ml of 0.5% (w/v) hexadecyltrimethylammonium bromide (HTAB) [4]. The skin site was ground up with a polytron homogenizer (Brinkmann Instruments, Westbury, NY) at highest setting for 15 sec. Tissue debris was removed by centrifugation at 800 *g* at room temperature for 10 min, and the supernatant fraction was used for MPO activity determination. The volume of the extract after homogenization and centrifugation was 10 ml ± 5%. All extractions were performed at room temperature; MPO activity in the supernatant fraction was stable at room temperature for several days. Skin extracts were not frozen or cooled since this resulted in precipitation of the HTAB.

MPO was determined as described in the *Worthington Enzyme Manual* [5] using *O*-dianisidine as hydrogen donor. Results were expressed as μmoles dianisidine oxidized per hr per total skin extract. The PBS-injected site on repeated assay was found to contain zero or very low levels of MPO. Only the antibody-injected skin site was, therefore, routinely extracted and assayed for MPO activity.

Histological sections were prepared from skin sites fixed in buffered formalin solution, embedded in paraffin and stained with hematoxylin and eosin.

RESULTS

Figure 1 shows the development of the edema, as measured by wet weight, and the cellular infiltrate, as measured by extractable MPO, as a function of time in the developing RPA in non-drug dosed rats. The maximal edematous response occurred 4–6 hr post-challenge in the skin and thereafter showed a slow decline. The extractable MPO lagged behind the edema response but also reached maximal values at 4–6 hr post-injection, declining slowly thereafter. Drug effects were measured at 4 hr post-challenge, which is the time of the expected maximal response and also the point of least variability in the edema and MPO values.

The results in Tables 1–3 show the effects of various anti-inflammatory drugs on the RPA. The steroidal anti-inflammatory drug dexamethasone (Table 1) inhibited the infiltration of cells in a dose-dependent manner with an ED_{50} of 0.02 mg/kg. Dexamethasone also significantly inhibited the edematous response. Because of the effect of dexamethasone in inhibiting both edema and MPO, dexamethasone was routinely included in the routine evaluation of the RPA at a dose of 0.07 mg/kg.

The data in Tables 2 and 3 show the effects of non-steroidal anti-inflammatory drugs on the RPA. The conclusion drawn from these studies is that the non-steroidal anti-inflammatory agents were much more effective as inhibitors of PMN accumulation than as inhibitors of edema. Indomethacin, for example (Table 2), inhibited the cellular component of the response between 35 and 47% (which was not dose related over a range of 1.0 to 10 mg/kg), whereas no effect was seen on the formation of edema at the same dose levels. Ibuprofen, sulindac, phenylbutazone, and aspirin (Tables 2 and 3) had similar effects of inhibiting cell recruitment but not inhibiting edema. Phenylbutazone (Table 3) and aspirin (Table 3) at very high doses (150 mg/kg and 300 mg/kg respectively) showed significant inhibition of edema and, interestingly, the "ceiling inhibition" of MPO at the same site was not increased by these high levels of drug. One exception was naproxen (Table 3) which at 30 mg/kg showed 45% inhibition

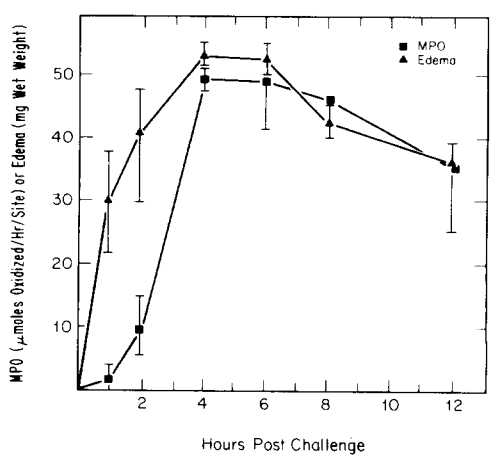

Fig. 1. Development of edema measured as wet weight and cell infiltration as measured by myeloperoxidase activity in the reverse passive Arthus reaction in the rat. Each point represents the mean of six animals. Bars represent standard deviations.

Table 1. Effect of dexamethasone on edema (wet wt) and PMN accumulation (MPO levels) in the reverse passive Arthus reaction in the rat*

Dose of dexamethasone (mg/kg, p.o.)	Edema (mg wet wt)	MPO (μmoles substrate oxidized/hr/site)
Control	49.0 ± 6.5	49.1 ± 17
0.025	30.5 ± 18.3 (38%)†	25.4 ± 16.4 (48%)†
0.05	22.8 ± 8.5 (54%)†	13.9 ± 5.4 (72%)†
0.100	26.2 ± 8.4 (47%)†	9.6 ± 4.5 (81%)†

* Results are expressed as means ± standard deviation (N = 6).
† Percent change is statistically significant ($P < 0.05$).

Table 2. Effects of non-steroidal anti-inflammatory drugs on edema (wet wt) and PMN accumulation (MPO levels) in the reverse passive Arthus reaction in the rat*

Treatment	Dose (mg/kg, p.o.)	Edema (mg wet wt)	MPO (μmoles oxidized/hr/site)
Experiment A:			
Control		41.5 ± 12.8	45.2 ± 9.7
Dexamethasone	0.07	24.1 ± 7.3 (42%)†	14.4 ± 10.2 (68%)†
Indomethacin	1.0	47.0 ± 7.8 (−13%)	29.3 ± 8.5 (35%)†
Indomethacin	3.0	37.7 ± 1.32 (9%)	24.1 ± 4.2 (47%)†
Indomethacin	10.0	42.2 ± 17.2 (−2%)	26.7 ± 7.4 (41%)†
Experiment B:			
Control		51.9 ± 11.3	69.0 ± 16.4
Dexamethasone	0.07	30.5 ± 8.6 (41%)†	31.6 ± 2.5 (54%)†
Ibuprofen	15.0	61.6 ± 17.3 (−19%)	49.6 ± 15.6 (28%)
Ibuprofen	30.0	45.3 ± 10.6 (13%)	47.0 ± 10.1 (32%)†
Ibuprofen	60.0	52.2 ± 10.2 (1%)	40.5 ± 8.8 (41%)†
Experiment C:			
Control		34.7 ± 5.8	37.3 ± 6.3
Dexamethasone	0.07	21.6 ± 22.1 (38%)†	11.4 ± 7.8 (69%)†
Sulindac	7.5	41.6 ± 14.0 (−20%)	45.9 ± 10.5 (−23%)
Sulindac	15.0	30.4 ± 6.7 (12%)	32.4 ± 9.3 (13%)
Sulindac	50.0	28.9 ± 4.2 (17%)	20.3 ± 6.8 (46%)†
Sulindac	100.0	33.1 ± 12.0 (5%)	15.2 ± 10.9 (59%)†
Experiment D:			
Control		49.0 ± 6.5	49.1 ± 17.0
Benoxaprofen	10	52.3 ± 4.2 (−8%)	51.7 ± 7.0 (−5%)
Benoxaprofen	30	44.8 ± 6.0 (9%)	49.1 ± 12.1 (0%)
Benoxaprofen	90	42.9 ± 13.3 (12%)	51.8 ± 21.1 (−6%)

* Results are expressed as means ± standard deviation (N = 6).
† Percent change is statistically significant ($P < 0.05$).

Table 3. Effects of non-steroidal anti-inflammatory drugs on edema (wet wt) and PMN accumulation (MPO levels) in the reverse passive Arthur reaction in the rat*

Treatment	Dose (mg/kg, p.o.)	N	Edema (mg wet wt)	MPO (μmoles oxidized/hr/site)
Control		18	53.6 ± 11.0	74.8 ± 20.3
Dexamethasone	0.07	18	28.1 ± 13.7 (48%)†	30.3 ± 18.5 (60%)†
Phenylbutazone	10	6	46.6 ± 12.5 (13%)	59.7 ± 15.5 (20%)
	30	12	47.5 ± 17.1 (11%)	38.8 ± 19.6 (48%)†
	90	12	44.5 ± 14.6 (17%)	33.7 ± 18.5 (55%)†
	150	6	41.7 ± 11.8 (22%)†	34.9 ± 16.2 (53%)†
Aspirin	33	6	56.3 ± 11.1 (−5%)	75.0 ± 34.9 (0%)
	100	6	57.5 ± 11.9 (−7%)	51.7 ± 18.0 (31%)†
	300	6	38.0 ± 7.8 (29%)†	36.5 ± 9.5 (39%)†
Naproxen	3	6	41.7 ± 10.4 (22%)†	60.6 ± 9.9 (19%)
	10	6	44.9 ± 8.4 (17%)	39.0 ± 12.2 (49%)†
	30	12	39.2 ± 11.1 (27%)†	40.9 ± 12.0 (45%)†
	60	6	31.6 ± 12.8 (41%)†	29.8 ± 8.4 (60%)†
	120	6	28.6 ± 8.2 (47%)†	24.2 ± 8.3 (67%)†
BW755C	5	6	46.4 ± 15.3 (13%)	82.7 ± 42.2 (−11%)
	20	6	54.3 ± 16.1 (−1%)	59.9 ± 18.0 (20%)
	60	5	49.8 ± 6.6 (7%)	43.7 ± 14.0 (42%)†

* Results are expressed as means ± standard deviation.
† Percent change is statistically significant ($P < 0.05$).

of MPO levels and a statistically significant 27% inhibition of edema with a good dose–response relationship.

Benoxaprofen is claimed to be a lipoxygenase inhibitor with only low cyclooxygenase inhibitor activity [6]. In experiment D of Table 2, the activity of this compound was very weak against both edema and MPO levels. BW755C (Table 3), which is equipotent as a lipoxygenase and cyclooxygenase inhibitor [7], was active in the RPA at 60 mg/kg but, like the other pure cyclooxygenase inhibitors, only inhibited MPO levels.

Attempts were made to confirm histologically the results obtained using MPO as the marker for PMN accumulation. Examination of skin sections from the RPA reaction sites showed an accumulation of large numbers of cells within the deep dermis (Fig. 2), which was composed of a mixed population of PMNs and mononuclear cells (mainly PMNs). There was no evidence of hemorrhage within these reaction sites. Skin sections from animals dosed with indomethacin did indeed show that there were fewer PMNs at the site of the Arthus reaction (Fig. 3), thus tending to confirm the results obtained using the MPO determinations. Interestingly, in the indomethacin-treated animals the accumulation of mononuclear cells was found to be largely unaffected. Indomethacin treatment, therefore, apparently changed the infiltrating cell type from predominantly PMN to that of a mononuclear cell by selectively decreasing the number of PMNs accumulating at the site. Figure 4 shows that dexamethasone treatment dramatically reduced the number of infiltrating cells and, as with indomethacin, the remaining infiltrating cell was found to be the mononuclear cell. Whereas histology yields valuable information on the types of infiltrating cells, biochemical methods as described in this report are necessary for adequate quantitation of the cellular response.

DISCUSSION

The data presented in this report show that non-steroidal anti-inflammatory drugs, which are cyclooxygenase inhibitors, inhibited the accumulation of cells at the site of the RPA at dose levels that had very little effect on edema.

The RPA has not been used extensively as a model system for studying anti-inflammatory drugs because, whilst steroids have shown good inhibitory activities, the effects of the non-steroidal anti-inflammatory drugs have been very poor in this system

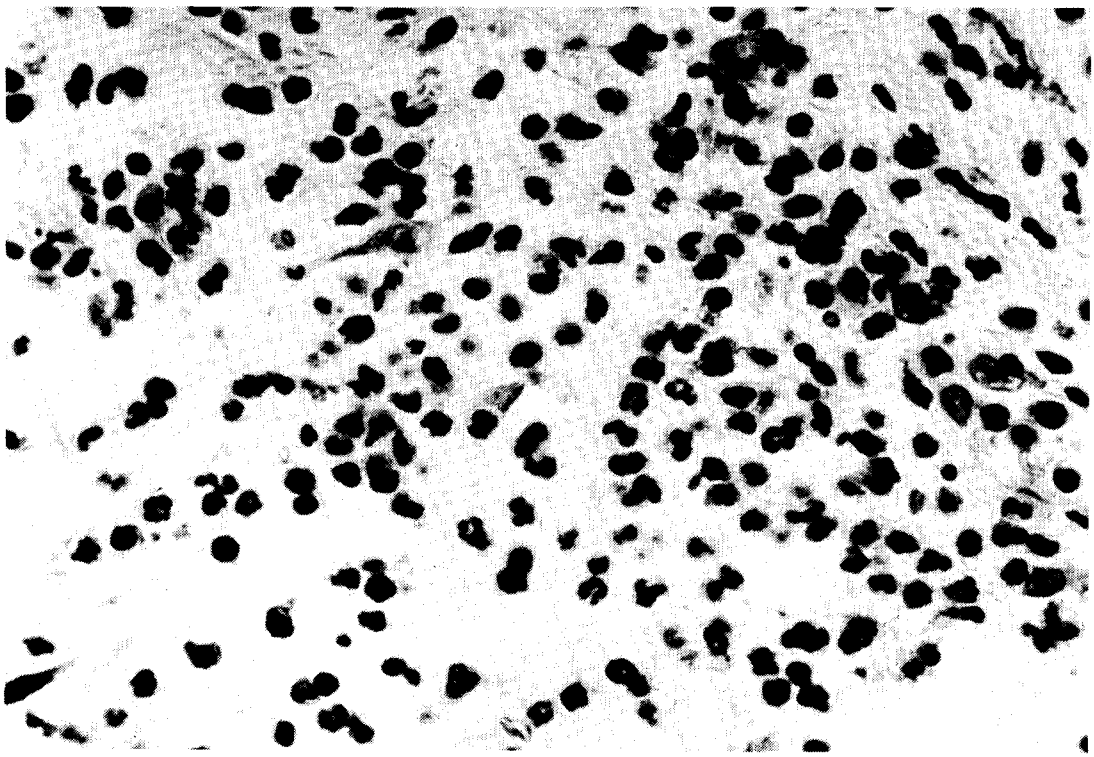

Fig. 2. H + E stained section through the site of the RPA reaction in a vehicle-dosed rat, 4 hr after challenge with antigen/antibody. Dense infiltration of inflammatory cells composed of a mixed population of PMNs and mononuclear cells. Magnification: × 531.25.

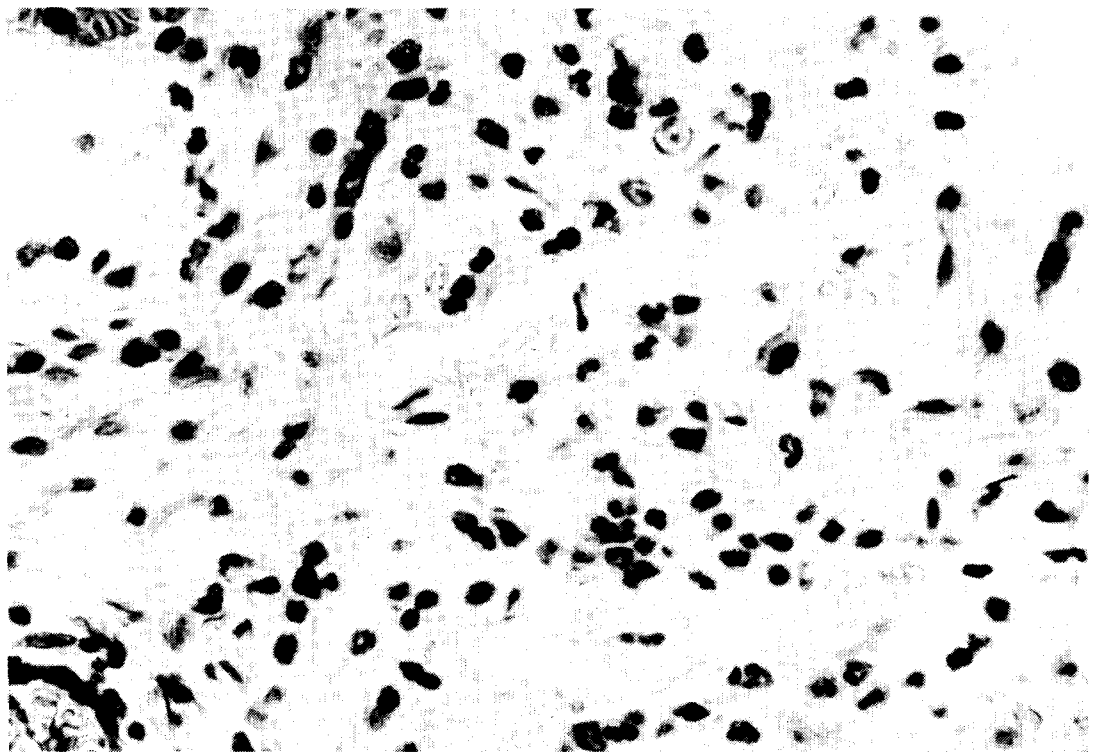

Fig. 3. H + E stained section through the site of the RPA reaction in a rat receiving 3 mg/kg indomethacin 1 hr before challenge with antigen/antibody. The infiltration of PMNs to the site of the reaction, 4 hr after challenge, is very much reduced, whereas the mononuclear cell infiltration is largely unaffected. Magnification: × 531.25.

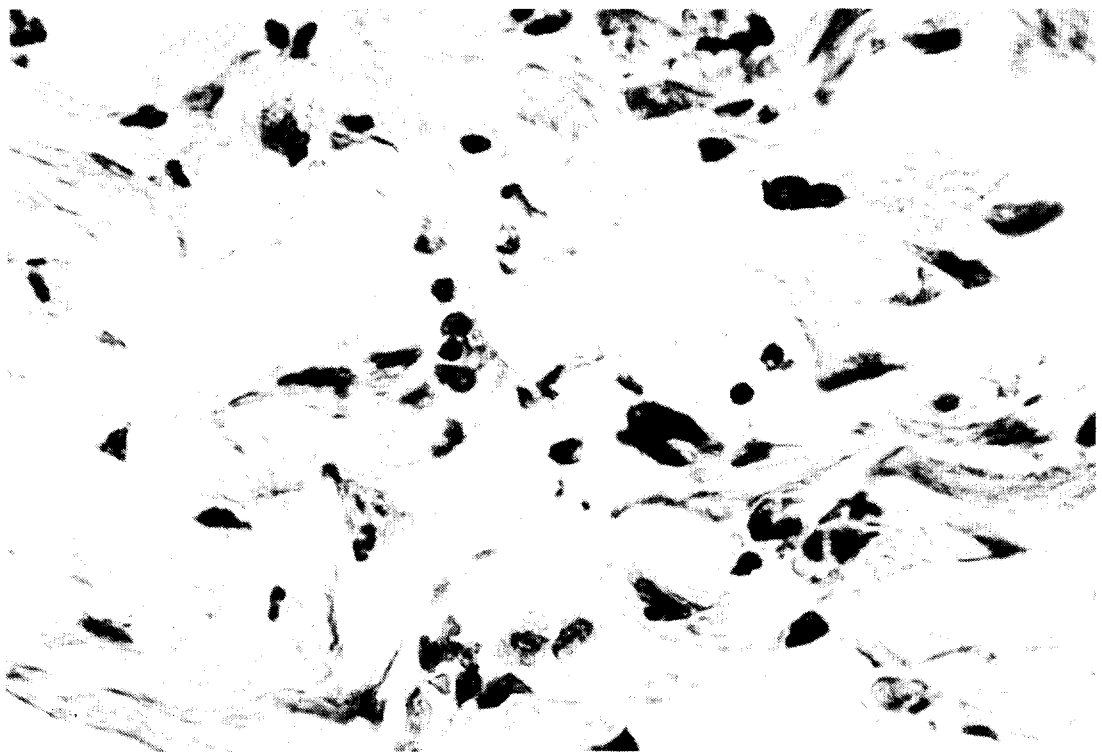

Fig. 4. H + E stained section through the site of the RPA reaction in a rat receiving 0.07 mg/kg dexamethasone 1 hr before challenge with antigen/antibody. There is a large reduction in the number of infiltrating PMNs and mononuclear cells at the site of the reaction measured 4 hr after challenge. Magnification: × 531.25.

[8, 9]. Most studies have examined only one component of this reaction, namely the formation of edema as measured by dye leakage or other methods [8, 9]. The cellular component of the reaction has been largely ignored probably due to the necessity for time-consuming histological studies and the difficulty of making quantitative estimates of the number of infiltrating cells from histologic preparations.

The RPA has been shown to be a complement-dependent reaction characterized by edema formation together with the accumulation of large numbers of PMNs at the site of the lesion [1, 10]. Complement depletion with cobra venom factor prevents the accumulation of PMNs at the site of an RPA. Similarly, depletion of PMNs with nitrogen mustard inhibits the development of edema [11]. The two processes, edema formation and PMN accumulation, are thus mutually dependent.

In this study, MPO was used as a marker enzyme for measuring PMN accumulation at the site of the RPA, using a method similar to that described by Bradley *et al.* [4] in skin inflammed by the intradermal injection of staphylococci. Non-involved skin contains virtually no extractable MPO, and mature mononuclear cells are known to contain only low levels of this enzyme. The extractable MPO is, therefore, a simple measure of the infiltrating PMNs at a site of inflammation.

Indomethacin is an inhibitor of MPO activity *in vitro* (unpublished results). It can be argued, therefore, that the decreased MPO activity found in skin from animals treated with indomethacin was due to the presence of residual indomethacin in the skin extract resulting in direct inhibition of the enzyme. Experiments showed (unpublished results) that addition of indomethacin to skin extracts did not inhibit MPO at levels below 10^{-4} M. Using our standard extraction procedure for MPO, it would be impossible to reach a level of 10^{-4} indomethacin in an 8 mm skin biopsy extracted into 10 ml of 0.5% HTAB. The decrease in MPO activity seen with indomethacin treatment was, therefore, a reflection of decreased infiltrated cells and not due to inhibition of the enzyme by residual indomethacin at the skin site.

Using the MPO technique, it was shown that cyclooxygenase inhibitors inhibited the infiltration of PMNs, whereas the effect on the edematous response at the same drug dose was very much less or entirely absent. Dexamathasone, on the other hand, reduced both cell infiltration and edema formation. In the light of this information it is important to know the nature of the mediators of both cell infiltration and edema at the site of the RPA reaction.

Unquestionably, complement activation products play a major role in causing cell infiltration due to the deposition of immune complexes [12]. C5a and its stable derivative C5a des Arg are found in serum treated with immune complexes [13], and these derivatives are powerful chemoattractants for PMN leukocytes *in vitro* [14]. There is no evidence in the literature, however, that the cyclooxygenase inhibitors which have been shown in this report to interfere

with cell recruitment in the RPA do so by interfering with the activation of complement components. Our results suggest that products of the cyclooxygenase pathway are important mediators in the chemoattraction of leukocytes in the RPA. Recent reports [15, 16] have indicated that edema formation caused by C5a injected locally in the rabbit has a requirement for PGE_2 and that, in the absence of PGE_2, C5a is inactive in edema formation. Therefore, in the present context it is possible that, *in vivo*, prostaglandins are also required for the chemotactic properties of C5a, and that this is why inhibitors of cyclooxygenase are effective in reducing leukocyte accumulation.

It is interesting that BW755C and benoxaprofen, which are both lipoxygenase inhibitors [6, 7] have relatively weak activities in this assay, suggesting that the products of the lipoxygenase pathway of arachidonic acid metabolism play a relatively small part in causing cell accumulation in the RPA.

One explanation for these observations is that the edematous response in the RPA may be dependent on the presence of PMNs, as seems to be the case with a carrageenan- or a zymosan-induced inflammatory edematous response [13, 14]. Although the non-steroidal anti-inflammatory drugs did inhibit the infiltration of PMNs, the inhibition was never 100%, so there may still have been sufficient numbers of PMNs at the site to induce a full edematous response. Contrary to this line of reasoning are the observations that inhibition of enzyme levels to 47% with indomethacin (Table 2) had no effect on the edema; whereas inhibition of enzyme levels to 68% (Table 2) with dexamethasone resulted in a 42% inhibition of edema in the same test.

The role of cyclooxygenase products of arachidonic acid oxidation in the accumulation of cells in the RPA remains to be clarified. The RPA reaction as described here forms a useful system for the evaluation of non-steroidal anti-inflammatory drugs and a novel system for the analysis of mediators of the inflammatory response due to the deposition of immune complexes within tissues.

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