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Comparative effects of inhibitors of the lipoxygenase enzyme system on leucocyte chemotaxis

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The chemotactic response, towards zymosan activated serum (ZAS), of elicited peritoneal exudate leucocytes was assessed in-vitro after treatment with inhibitors of the lipoxygenase enzyme pathway. Leucocytes from both rat and guinea-pig were used. BW755C and benoxaprofen reduced the chemotaxis of mononuclear cells from both species at 10^{-4} M. Nordihydroguaiaretic acid (NDGA) at $7{\cdot}5 \times 10^{-6}$ M depressed the chemotaxis of rat mononuclear cells but failed to modify the response of guinea-pig mononuclear cells. NDGA, BW755C and benoxaprofen, at concentrations that inhibit the lipoxygenase enzyme pathway in-vitro, were ineffective in reducing the chemotactic response of rat PMN. All three agents potentiated guinea-pig PMN chemotaxis, to a greater or lesser extent, the most pronounced effect being with NDGA when increased PMN migration was accompanied by an increased detachment of cells from the lower surface of the filters. The effects of these agents on both cellular chemotaxis and adherence are not necessarily related to their inhibitory effects on the lipoxygenase enzyme pathway.

The mechanism by which leucocytes respond to chemoattractants is complex and involves many aspects of cellular biochemistry. It is reasonable to suppose, therefore, that the mechanism of action of agents that inhibit cellular chemotaxis may be diverse and not necessarily due to any single action on the cell. However, it has been suggested that endogenous mono-hydroxy-eicosatetraenoic acids, particularly 5- and 11-HETEs, derived from the lipoxygenation of intracellular arachidonic acid are closely associated with cell movement. Depletion of intracellular levels of 5- and 11-HETEs by inhibitors of the lipoxygenase enzyme pathway has been shown to result in a diminished cellular chemotactic response (Goetzl 1980). Also, inhibition of PMN chemotaxis by eicosatetraynoic acid ETYA, but not by indomethacin (Malmsten et al 1980) and by nordihydroguaiaretic acid (NDGA) (Showell et al 1980) further suggests that this pathway of arachidonic acid metabolism may be important in cell locomotion.

More recent data implicate the involvement of endogenous lipoxygenase products in the migration of rat aortic smooth muscle cells in response towards platelet-derived growth factor (PDGF) (Nakao et al 1983).

The initial consequence of binding of a chemoattractant to receptors on the surface of the cell membrane, is an influx of Ca^{2+} and Na^{+} ions (Gallin et al 1978). Additional alterations in intracellular Ca^{2+} levels eventually create an environment which is favourable for assembly of the intracellular microtubule system which is implicated in oriented cell movement. It has been demonstrated that the mono-hydroxy-eicosatetraenoic acids, 5-, 11-, 12-HETE and 5:12-dihydroxyeicosatetraenoic acid (leukotriene B_4) increase the uptake of Ca^{2+} into polymorphonuclear leucocytes (PMN). In addition, LTB_4 also initiates the mobilization of intracellular Ca^{2+} (Naccache et al 1981). Although the inhibitory effects of the lipoxygenase inhibitor NDGA have been effectively demonstrated using human peripheral blood PMN (Goetzl 1980), to our knowledge there have been no reports on the effect of this agent on the chemotactic response of PMN from other species nor on the chemotactic response of mononuclear cells in-vitro.

In the following experiments, the role of intracellular lipoxygenases in cell movement has been explored in greater depth and the chemotactic responses of elicited leucocytes from both rat and guinea-pig, in the presence of the lipoxygenase inhibitors, NDGA, BW755C (3-amino-1-[*m*-(trifluoromethyl)phenyl]pyrazoline HCl and benoxaprofen (Walker & Dawson 1979; Harvey et al 1983) have been examined.

MATERIALS AND METHODS

Rat leucocytes were elicited by intraperitoneal injection of 2 ml 2% oyster glycogen (Koch Light Laboratories) in 0·9% NaCl (saline). Cellular exudates were harvested 24 h later by a further injection of 20 ml ice-cold Gey's Balanced Salt Solution

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(Gey's BSS) containing 5 units ml^{-1} preservative-free heparin (Evans Medical). Exudates were withdrawn directly into a sterile syringe through the peritoneal wall. Leucocyte suspensions consisted of 60–70% mononuclear cells, the remainder being PMN.

Where relatively pure PMN were required, these were elicited in the peritoneal cavity by injection of 1 ml homologous rat serum. Cell suspensions comprised 70–80% PMN when harvested 4 h after serum injection.

Guinea-pig mononuclear cells were elicited by intraperitoneal injection of 15 ml 1% glycogen in saline and recovered 72 h later by the method described above. Leucocyte suspensions elicited in this way contained 85–90% mononuclear cells. Guinea-pig PMN were elicited by intraperitoneal injection of 12 ml 2% casein (Hopkin and Williams) in saline. PMN comprised 75–85% of the total number of leucocytes recovered 17 h later.

All cell suspensions were centrifuged at 1000 rev min^{-1} for 5 min and the cell pellets resuspended in Gey's BSS. No additional separative procedures were carried out.

Chemotaxis assay

The method used was a modification of that described by Wilkinson (1974). Polycarbonate membranes (Uni-Pore: Bio-Rad) of 5·0 μm pore size were attached to the machine-ground ends of glass tubes using a non-cytotoxic adhesive (UHU-Lingner Fischer, GmBH). The tubes were suspended in glass beakers containing 5 ml of the chemotaxin; one beaker serving as the lower compartment for duplicate tubes. Zymosan-activated homologous rat serum (ZAS, 5% in Gey's BSS) was used as the chemotaxin and adjusted to pH 7·2 immediately before use.

Indicator cells were lightly centrifuged, resuspended in either Gey's BSS or in drug solution, at a concentration of $5{\cdot}0 \times 10^6$ cells ml^{-1} and preincubated in rolling tubes for 60 min at 37 °C. Following preincubation, cells were again washed and resuspended in Gey's BSS, or in the appropriate solution of drug, to give a final concentration of $5{\cdot}0 \times 10^6$ cells ml^{-1}. Aliquots of 0·2 ml of the cell suspension were placed in each of the tubes. The chambers were incubated for 120 min at 37 °C when either rat or guinea-pig mononuclear cells were used. For assay of the PMN chemotactic response, chamber incubation was reduced to 90 min. Cell viability on residual cell suspensions was assessed by dye exclusion using nigrosin at a concentration of 0·35% in saline.

Evaluation

The membranes were simultaneously fixed and detached from the tubes by immersion in 95% ethanol. Adherent leucocytes were stained with Mayers haematoxylin, cleared in 2-ethoxyethanol and xylene and mounted in DPX. Chemotaxis was evaluated by the lower surface counting method. Numbers of leuocytes migrating per microscope field were counted at a magnification of 630×, in 10 fields per filter. Between 4–6 filters were used for each estimation. Statistical analysis of the data was carried out using Student's *t*-test.

Quantitation of detaching leucocytes

The total number of PMN that had detached from each of the two filters per lower chamber was quantitated. Given that the mean area of cell coverage per filter was 27·0 mm^{-2} and the area of one microscope field (magnification 630×) was 0·03 mm^{-1}, the number of fields per filter was calculated to be 913. Hence the mean number of PMN detaching per field could be calculated. Such a calculation, however, was necessarily based on the assumption that PMN detach in equal numbers from each of the duplicate filters per lower chamber. In the following experiments, values for PMN adhering to the lower surface of the filter and PMN detaching from the filters have been combined and are expressed as 'total' migration.

RESULTS

Mononuclear cell chemotaxis

The chemotactic response of rat peritoneal mononuclear cells towards ZAS was significantly reduced following preincubation and resuspension of the cells in NDGA, BW755C and benoxaprofen (Table 1). BW755C and benoxaprofen, at 10^{-4} M, inhibited mononuclear cell chemotaxis by 71 and 67% respectively. NDGA, at $7{\cdot}5 \times 10^{-6}$ M reduced the chemotactic response of these cells by 47%.

Table 1. Effect of lipoxygenase inhibitors on the chemotactic response of rat mononuclear cells towards ZAS.

Treatment	Concn (M)	Mean no. mononuclear cells per field mean±s.e.m.	% Red[n]	$P<$
Controls		54·3 ± 1·5		
BW755C	10^{-4}	15·5 ± 1·2	71	0·001
Controls		43·3 ± 3·0		
Benoxaprofen	10^{-4}	14·2 ± 1·8	67	0·001
Controls		84·3 ± 4·1		
NDGA	$7{\cdot}5 \times 10^{-6}$	44·9 ± 4·7	47	0·001

The effects of these agents on the chemotaxis of mononuclear cells from guinea-pigs are shown in Tables 2 and 3. BW755C caused a substantial reduction (89%) in the chemotaxis of these cells at a concentration of 10^{-4} M, whereas NDGA at $7{\cdot}5 \times 10^{-6}$ M failed to modify cell movement (Table 2). Treatment of mononuclear cells with benoxaprofen resulted in a concentration-dependent reduction in chemotaxis at levels between $0{\cdot}5$–$1{\cdot}5 \times 10^{-4}$ M (Table 3).

Table 2. Effects of BW755C and NDGA on the chemotactic response of guinea-pig mononuclear cells towards ZAS.

Treatment	Concn (M)	Mean no. mononuclear cells per field Mean±s.e.m.	% Red^n	$P<$
Controls		$70{\cdot}5 \pm 1{\cdot}8$		
BW755C	10^{-4}	$8{\cdot}1 \pm 0{\cdot}6$	89	0·001
Controls		$138{\cdot}6 \pm 8{\cdot}8$		
NDGA	$7{\cdot}5 \times 10^{-6}$	$145{\cdot}7 \pm 6{\cdot}6$	−5	N.S.

On no occasion did mononuclear cells become detached from the lower surface of the filters.

PMN chemotaxis

The chemotactic response of 4 h serum-elicited peritoneal PMN from rats was not affected by treatment with either BW755C (Table 4) nor benoxaprofen (Table 5) at concentrations ranging from 1×10^{-5}–1×10^{-4} M. Detachment of PMN from the undersurface of the filters did not differ significantly from that of untreated cells.

Table 3. Effects of benoxaprofen on the chemotactic response of guinea-pig mononuclear cells towards ZAS.

Treatment	Concn (M)	Mean no. mononuclear cells per field Mean±s.e.m.	% Red^n	$P<$
Controls		$151{\cdot}1 \pm 4{\cdot}9$		
Benoxaprofen	$0{\cdot}5 \times 10^{-4}$	$94{\cdot}5 \pm 6{\cdot}7$	38	0·001
Benoxaprofen	1×10^{-4}	$89{\cdot}1 \pm 8{\cdot}0$	41	0·001
Benoxaprofen	$1{\cdot}5 \times 10^{-4}$	$72{\cdot}7 \pm 4{\cdot}9$	52	0·001

NDGA, at 2·5 and $7{\cdot}5 \times 10^{-6}$ M, also failed to modify the migration of these cells, but at $2{\cdot}5 \times 10^{-5}$ M, gave a significant inhibition of 80%. Detachment of PMN was significantly increased (45%) above control values by NDGA at the lower level of $2{\cdot}5 \times 10^{-6}$ M (Table 6).

The profile of activity of the three agents against the chemotaxis of guinea-pig PMN was essentially similar in that at the concentrations tested (Table 7) neither BW755C, NDGA, nor benoxaprofen suppressed the total migration of these cells. Migration was, in-fact, significantly increased over controls after treatment with BW755C and NDGA, by 29% and 100% respectively. Associated with these effects were significant increases in the numbers of PMN detaching from the lower surface of the filters; the most dramatic effect being observed in the presence of NDGA at $7{\cdot}5 \times 10^{-6}$ M (Table 7). PMN detachment was not affected by benoxaprofen.

Table 4. Effects of BW755C on the chemotactic response of rat PMN towards ZAS.

Treatment	Concn (M)	Mean no. PMN per field mean±s.e.m.	% Red^n	$P<$
Controls		$320{\cdot}2 \pm 11{\cdot}3$		
BW755C	1×10^{-5}	$307{\cdot}5 \pm 16{\cdot}5$	4	N.S.
BW755C	$3{\cdot}3 \times 10^{-5}$	$279{\cdot}7 \pm 12{\cdot}6$	13	0·05
Controls		$341{\cdot}3 \pm 13{\cdot}9$		
BW755C	1×10^{-4}	$306{\cdot}1 \pm 24{\cdot}9$	10	N.S.

Table 5. Effects of benoxaprofen on the chemotaxis of rat PMN.

Treatment	Concn (M)	Total migration PMN per field Mean±s.e.m.	% Red^n	$P<$
Controls		$336{\cdot}0 \pm 18{\cdot}0$		
Benoxaprofen	1×10^{-5}	$316{\cdot}6 \pm 14{\cdot}2$	6	N.S.
Benoxaprofen	$3{\cdot}3 \times 10^{-5}$	$333{\cdot}8 \pm 18{\cdot}2$	1	N.S.
Benoxaprofen	1×10^{-4}	$365{\cdot}7 \pm 21{\cdot}2$	−9	N.S.

DISCUSSION

In the experiments described, the lipoxygenase inhibitors NDGA, BW755C and benoxaprofen effectively reduced the chemotactic response of rat peritoneal mononuclear cells towards ZAS, although they exhibited differing profiles of activity against mononuclear cells derived from guinea-pigs. In contrast, all three agents failed to modify the chemotactic response of rat PMN at concentrations which are known to inhibit the synthesis and release of intracellular lipoxygenase-derived products of arachidonic acid from PMN in-vitro (Harvey & Osborne 1983). NDGA suppressed PMN chemotaxis at levels far in excess of these ($2{\cdot}5 \times 10^{-5}$ M).

A preferential effect against mononuclear cell chemotaxis has previously been demonstrated by benoxaprofen with leucocytes derived from rats (Meacock & Kitchen 1979) guinea-pigs and volunteers (Meacock et al 1982). Such effects have also been confirmed by Goetzl & Valone (1982) using human peripheral blood leucocytes. Information

Table 6. Effects of NDGA on the chemotaxis and adherence of rat PMN.

Treatment	Concn (M)	PMN detaching per field Mean ± s.e.m.	% Red[n]	P<	Total migration PMN per field Mean ± s.e.m.	% Red[n]	P<
Controls		202·5 ± 14·9			320·2 ± 11·3		
NDGA	$2·5 \times 10^{-6}$	292·9 ± 13·6	−45	0·01	342·8 ± 18·4	−7	N.S.
NDGA	$2·5 \times 10^{-5}$	65·1 ± 9·5	68	0·001	65·3 ± 9·5	80	0·001
Controls		284·8 ± 40·5			382·5 ± 36·3		
NDGA	$7·5 \times 10^{-6}$	321·3 ± 20·9	−13	N.S.	357·7 ± 20·5	7	N.S.

Table 7. Effects of lipoxygenase inhibitors on the chemotactic response of guinea-pig PMN towards ZAS.

Treatment	Concn (M)	PMN detaching per field Mean ± s.e.m.	% Red.	P<	Total migration PMN per field Mean ± s.e.m.	% Red.	P<
Controls		80·3 ± 9·7			255·7 ± 9·9		
BW755C	10^{-4}	112·3 ± 5·2	−40	0·05	330·2 ± 19·4	−29	0·05
Controls		28·5 ± 9·9			297·3 ± 54·3		
NDGA	$7·5 \times 10^{-6}$	339·5 ± 50·2		0·02	594·9 ± 32·6	−100	0·02
Benoxaprofen	10^{-4}	20·9 ± 4·4	27	N.S.	321·6 ± 4·5	−8	N.S.

concerning the effects of NDGA and BW755C on mononuclear cell chemotaxis is not available, although it would appear from the data presented here, that these particular inhibitors of lipoxygenase also exert a preferential effect on the chemotaxis of mononuclear cells derived from rats. Such differential effects against rat leucocytes may relate to differences in the binding capacity of leucocytes for these agents. It has been shown that benoxaprofen demonstrates both high and low affinity binding to rat leucocytes, but mononuclear cells possess more binding sites overall for the drug than PMN (Meacock et al 1982). Differences in the sizes of these leucocytes did not account for the observed differences in binding. Similar studies have not been carried out using NDGA or BW755C.

The inability of these agents to modify PMN chemotaxis was also demonstrated using guinea-pig PMN, and in the case of BW755C and NDGA, significant potentiations in PMN migration were observed. In both cases, the increased migration was accompanied by a substantial increase in the numbers of PMN becoming detached from the lower surface of the filters. The most pronounced effect was observed in the presence of NDGA and appeared not to be restricted only to guinea-pig cells since similar effects on cell adherence were also evident when rat PMN were used, although effects were less dramatic. To what extent the increased detachment of NDGA-treated cells contributed to the overall increase in PMN migration (or vice versa) is difficult to establish.

NDGA has been shown to effectively inhibit arachidonic acid-induced aggregation of both human and rat PMN in-vitro (Ford-Hutchinson et al 1979). It was later shown, however, that the aggregatory activity was, in fact, mediated by LTB_4 and that lipoxygenase inhibitors reduced this activity (Ford-Hutchinson et al 1980). It is also well established that the chemotactic peptide C5a promotes the formation of a hyperadherent cell membrane (O'Flaherty et al 1976) and increases the adherence of these cells to inert substrata. In addition, C5a induces aggregation of PMN (Craddock et al 1977; Damerau et al 1980) and it has been demonstrated that NDGA also inhibits the aggregation of PMN induced by ZAS (J. R. Walker, personal communciation). Since cell-aggregation and adherence may, in some respects, be associated phenomena (Craddock et al 1979) it is possible that NDGA may be inhibiting C5a-mediated PMN adherence to the undersurface of the filters. This being so, however, it is surprising that neither BW755C nor benoxaprofen inhibited PMN adherence at concentrations which are known to inhibit the synthesis and release of lipoxygenase products of arachidonic acid. It would appear, therefore, that effects on cell adherence cannot be attributed entirely to lipoxygenase inhibition.

In addition to effects of NDGA on cell adherence, differences in species specificity may also account for the divergent effects of this agent on the chemotaxis of guinea-pig mononuclear cells. However, further information on the effects of NDGA on the release of lipoxygenase products from guinea-pig and rat

mononuclear cells is needed in order to explain such variations in effect.

From the available data, however, it is concluded that the lipoxygenase inhibitors NDGA, BW755C and benoxaprofen, demonstrated effects against leucocyte chemotaxis and adherence which were not consistent with a common mechanism of action for this particular class of drug.

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