Interference of anti-inflammatory and anti-asthmatic drugs with neutrophil-mediated platelet activation: singularity of azelastine

¹Patricia Renesto, Viviane Balloy, B. Boris Vargaftig & Michel Chignard

Unité de Pharmacologie Cellulaire, Unité associée Institut Pasteur/INSERM n° 285, 25 rue du Dr Roux, 75015 Paris, France

1 The capacity of various drugs (acetylsalicylic acid (ASA), ketoprofen, diclofenac, piroxicam, BW 755C, BW A4C, nedocromil sodium and azelastine) to inhibit human polymorphonuclear neutrophil (PMN)-mediated platelet activation was investigated. In this model, stimulated PMN release cathepsin G (Cat G), a serine proteinase which, in turn, induces platelet activation.

2 Among the different tested drugs, azelastine (100 μ M for 1 min) was the only one able to prevent platelet aggregation. The cyclo-oxygenase inhibitors were all inactive, although used at effective concentrations as judged by inhibition of thromboxane B₂ (TxB₂) formation. Inhibition of platelet aggregation by azelastine was concentration-dependent, the range of active concentrations being of 20–70 μ M. Release from platelets of 5-hydroxytryptamine was also inhibited at 30 μ M and above, but never reached 100%.

3 The inhibition by azelastine is due to an effect on both cells. Indeed, β -glucuronidase release from activated PMN and platelet activation by purified Cat G were both affected.

4 However, used at high concentrations (>100 μ M) azelastine was toxic since it released significant amounts of lactate dehydrogenase (LDH) from PMN and platelets.

5 These results show the capacity of azelastine, an anti-allergic and anti-asthmatic compound, to inhibit the cell-to-cell communication between PMN and platelets, an effect which may be relevant for its therapeutic efficacy or for a new application in diseases in which PMN and platelets are involved.

Keywords: Neutrophils; platelets; azelastine; inflammation; anti-asthmatic drugs; anti-inflammatory drugs.

Introduction

Agents such as N-formyl-Met-Leu-Phe (FMLP) or C5a induce the release from polymorphonuclear neutrophils (PMN) of cathepsin G (Cat G), a serine proteinase which induces, in turn, activation of nearby platelets (Chignard *et al.*, 1986; Selak *et al.*, 1988; Renesto *et al.*, 1990; Ferrer-Lopez *et al.*, 1990; Del Maschio *et al.*, 1990). This is reminiscent of different experimental acute inflammatory states evoked in laboratory animals. Thus, an intradermal injection of FMLP in the rabbit, triggers platelet deposition, a phenomenon which is absent in PMN-depleted but platelet-sufficient animals (Issekutz *et al.*, 1983). A PMN-dependent platelet deposition has also been observed during occlusion-reperfusion injury in the dog (Bednar *et al.*, 1985). In a PMN-mediated model of subendothelial immune complex glomerulonephritis, it has been shown that platelets mediate glomerular injury and proteinuria (Johnson *et al.*, 1988). In models of adult respiratory distress syndrome (ARDS), not only PMN are involved but also platelets participate (Tvedten *et al.*, 1985). In man, the participation of PMN (Warszawski *et al.*, 1986) and platelets (Heffner *et al.*, 1987) in lung injury observed during the ARDS is very likely. It has also been suggested that PMN contribute to immediate hypersensitivity (Baggiolini & Dewald, 1985; Weiss, 1989), and mediators released from these cells may be involved in allergic asthma (Durham *et al.*, 1984; Kay, 1987). Activated platelets are also potential contributors (Page, 1989). Moreover, they synthesize biologically active substances such as thromboxane A₂, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (Marcus *et al.*, 1988), or platelet-activating factor (PAF) (Chignard *et al.*, 1979) which are relevant to allergic inflammation.

Thus, such a cell-to-cell communication may contribute to various inflammatory states, and its inhibition may be relevant for therapy. In this paper, we have investigated whether different anti-inflammatory drugs (ASA, ketoprofen, diclofenac, piroxicam, BW 755C and BW A4C) or anti-asthmatic and/or anti-allergic drugs (nedocromil sodium, azelastine) might inhibit PMN-mediated platelet activation. Azelastine, a

new therapeutic agent with anti-asthmatic and anti-allergic properties used in the treatment of rhinitis (Meltzer *et al.*, 1988) and asthma (Spector *et al.*, 1987; Kemp *et al.*, 1987), was the only substance tested capable of preventing platelet aggregation and degranulation induced by stimulated PMN.

Methods

Preparation of human neutrophils

PMN were purified from blood of healthy donors collected on acid-citrate-dextrose (ACD) (1.5 ml for 10 ml of blood; composition, mM: trisodium citrate 85, citric acid 66, glucose 111) and heparin (20 i.u. ml⁻¹) as anticoagulant. Dextran (3% in saline) was added (0.5 volume for 1 volume of blood) and erythrocytes were allowed to sediment for 30 min. After centrifugation (350 g; 45 min) of the resulting supernatant through a Ficol-Paque gradient, the lysis of erythrocytes contaminating the pellet was performed with a specific 'lysis solution' (composition, mM: NH₄Cl 155, KHCO₃ 2.96, disodium EDTA 3.72). The resulting cell suspension was then gently inverted for 5 min and centrifuged (350 g; 10 min). After a wash in Hank's Balanced Salt Solution (HBSS), PMN were finally resuspended in HBSS in such a volume to obtain 10⁷ PMN per ml. The resulting PMN were 98.3 $\pm$ 1.2% pure, as estimated with Türk's stain, and 97.1 $\pm$ 2.6% viable as measured by the trypan blue exclusion method.

The concentrations of CaCl₂ and MgCl₂ were adjusted to 1.3 and 1.0 mM respectively, before each experiment.

Preparation of human washed platelets

Blood was collected as for PMN preparation. After a first centrifugation (180 g; 20 min), platelet rich plasma was collected and incubated with [¹⁴C]-5-hydroxytryptamine ([¹⁴C]-5-HT 0.05 μ Ci ml⁻¹ final concentration) for 30 min at 37°C (during the rest of the process the preparation was kept at this temperature). The cell suspension was then centrifuged (1450 g; 15 min) and the pellet was resuspended in a Tyrode buffer (composition, mM: NaCl 137.0, KCl 2.68, NaHCO₃

¹ Author for correspondence.

11.9, NaH_2PO_4 0.42, CaCl_2 2.0, MgCl_2 1.0, glucose 5.5, HEPES 5.0, BSA 0.35%; pH 7.4) supplemented with 10 nM prostacyclin and 50 i.u. ml^{-1} heparin. After a second centrifugation (1450g, 15 min) cells were washed again using the same medium but without heparin. Finally, the platelet concentration was adjusted to 4×10^8 cells per ml.

Aggregation and 5-hydroxytryptamine release determination

Platelet aggregation was studied with a Dual Chrono-Log Aggregometer (Chrono-Log-Corp., Hevertown, PA., U.S.A.). Platelets (250 μl) and PMN (250 μl) were incubated together for 5 min in siliconized test tubes, with stirring at 37°C, and in the presence of fibrinogen (0.77 mg ml^{-1}) and cytochalasin B (5 $\mu\text{g ml}^{-1}$). FMLP was then added, and the reaction was stopped 3 min later with 125 μl of a 'stopping solution' (composition, mM: EDTA 38.5, NaCl 68.8, formaldehyde 2.05%). Samples were rapidly centrifuged (13,000g; 2 min), and 400 μl of each supernatant were collected for [^{14}C]-5-HT release determination by aqueous counting scintillation (Counter 1212 Rackbeta, LKB-Wallac, Stockholm, Sweden). Aggregation was expressed as the percentage of change in light transmission, and 5-HT release as the percentage of total [^{14}C]-5-HT content of platelets.

β -Glucuronidase release determination

Aliquots of 1 ml of PMN (5×10^6 cells per ml) in HBSS-Tyrode (1:1) were preincubated for 4 min with cytochalasin B (5 $\mu\text{g ml}^{-1}$) and stirring at 37°C. Azelastine was then added, and 1 min later PMN were stimulated with 0.5 μM FMLP. The reaction was stopped after 3 min by placing the tubes on ice. Samples were then centrifuged (2,500g; 15 min), and 100 μl aliquots were collected and stored at -20°C in order to determine the amount of enzyme released in the supernatants. Pellets were resuspended in 1 ml of Triton X-100 0.2%, strongly vortexed, and incubated at 4°C overnight. Following centrifugation (2500g; 15 min) supernatants containing the enzyme which was retained in PMN were collected. Amounts of enzyme present in the two types of supernatants were determined spectrophotometrically, with 4-nitrophenyl- β -D-glucopyranosiduronic acid as substrate.

Release of β -glucuronidase was expressed as a percentage of the total β -glucuronidase content.

Lactate dehydrogenase determination

Aliquots of 2×10^8 platelets per ml or 5×10^6 PMN per ml (in HBSS-Tyrode 1:1) were placed in siliconized test tubes and after a 4 min preincubation period (as for aggregation procedure), azelastine was added at different concentrations. The reaction was stopped 4 min later with EDTA (5 mM final concentration). After centrifugation (2500g; 15 min), aliquots of the supernatants were stored at 4°C during the night and the pellets were resuspended in Triton X-100 0.1% in order to lyse the cells and to extract residual enzyme. Following an overnight incubation period, samples were centrifuged (2500g; 15 min) and supernatants collected. Enzyme concentration was determined with NADH as substrate. Release of lactate dehydrogenase (LDH) was expressed as percentage of the total LDH content.

Thromboxane B_2 and leukotriene B_4 measurements

Thromboxane B_2 (TxB_2) and leukotriene B_4 (LTB_4) were measured in supernatants of cells processed as for platelet aggregation, but without [^{14}C]-5-HT labelling. Briefly, for TxB_2 radioimmunoassay, samples were incubated with [^{125}I]- TxB_2 and anti- TxB_2 antibodies. Bound fractions were precipitated with polyethylene glycol 6000, and after centrifugation (2500g; 10 min) radioactivity of the pellet was counted in a gamma counter (Kontron Analytical MDA 312). For LTB_4

radioimmunoassay, [^3H]- LTB_4 and anti- LTB_4 antibodies were used. Addition of dextran-coated charcoal followed by a centrifugation (2500g; 10 min) allowed to separate unbound from bound fractions. Radioactivity in supernatants corresponding to the amount of radioactive free ligand was measured (Counter 1212 Rackbeta, LKB-Wallac, Stockholm, Sweden). Finally, TxB_2 and LTB_4 concentrations were determined by interpolation from standard curves.

Materials

Blood was obtained from the Centre National de Transfusion Sanguine, Paris, France. Dextran, PAF, cytochalasin B, FMLP, TxB_2 , and prostacyclin were purchased from Sigma Chemical Corp., St. Louis, MO, U.S.A. The Ficoll-Paque was from Pharmacia, Uppsala, Sweden. The Aqueous Counting Scintillant (ACS II), [^{14}C]-5-HT, and [^3H]- LTB_4 assay reagent system were from Amersham International, Amersham, Buckinghamshire. The LDH kit was purchased from Boehringer, Mannheim, Germany. Heparin was from Choay, Paris, France, and BSA from Euromedex, Strasbourg, France. Polyethylene glycol 6000 and 4- β -D-glucopyrasidonuronic acid were from Merck, Darmstadt, Germany. Antibodies and radiolabelled ligand for RIA of TxB_2 were supplied by Prof. F. Dray, INSERM 207, Institut Pasteur, Paris, France. Human fibrinogen was obtained from Kabi, Stockholm, Sweden, and was treated with diisopropyl fluorophosphate in order to eliminate coagulant contaminants. Cat G was purified from human PMN following the method of Baugh & Travis (1976) modified by Martodam *et al.* (1979). ASA (Aspegic), was purchased from Synthelabo, Paris, France. Azelastine was a gift from Drs J.C. Venco, D. Perrisoud and J. Roux, Laboratoires Sarget, Merignac, France. Ketoprofen, diclofenac, and piroxicam were gifts from Dr B. Terlain, Rhône-Poulenc Santé, Vitry, France. N-(3-benzylcinnamyl) acetohydroxamic acid (BW A4C) and 3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline (BW 755C) were gifts from Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent. Nedocromil sodium was a gift from Drs K. Rainey and A. Norris, Fisons plc, Loughborough, Leicestershire.

Statistical analysis

Each figure is the mean $\pm$ s.d. of the number of experiments indicated in parentheses. The Student's *t* test for unpaired samples was used to evaluate the significance. Results were significant (*) when $P < 0.05$.

Results

Effects of anti-inflammatory and anti-asthmatic drugs on the PMN-mediated platelet activation

As shown in Table 1, the cyclo-oxygenase inhibitors, ASA (100 μM for 5 min), ketoprofen, diclofenac and piroxicam (all 10 μM for 1 min) inhibited by approx. 30% PMN-induced release of 5-HT from platelets and failed to affect PMN-induced platelet aggregation ($P > 0.05$). TxB_2 measurements indicated that the anti-cyclo-oxygenase activity of these substances was effective in these experimental conditions. Acetohydroxamic acids, BW 755C and BW A4C (100 μM for 5 min) which possess anti-lipoxygenase and anti-cyclo-oxygenase properties (Randall *et al.*, 1980; Tateson *et al.*, 1988) were used at concentrations which inhibited LTB_4 and TxB_2 production, respectively. BW A4C also failed to modify platelet aggregation and 5-HT release, and BW 755C reduced marginally platelet aggregation, 5-HT release being reduced by 50%. The anti-asthmatic drug, nedocromil sodium (Eady, 1986), had no effect on the system. In fact, azelastine (100 μM for

Table 1 Effects of different drugs on platelet activation induced by FMLP-stimulated PMN

Drug	Concentration (μM)	Aggregation	5-HT release	LTB_4 synthesis	TxB_2 synthesis
ASA	100	97.5 ± 5.4	72.9 ± 12.3	77.8 ± 10.3	$4.2 \pm 5.3^*$
Ketoprofen	10	100.5 ± 4.7	64.0 ± 10.6	100.6 ± 31.1	$2.8 \pm 2.8^*$
Diclofenac	10	91.9 ± 7.0	73.4 ± 10.9	88.1 ± 16.9	0^*
Piroxicam	10	96.9 ± 8.1	73.6 ± 13.9	98.0 ± 27.1	$0.9 \pm 1.9^*$
BW 755C	100	86.3 ± 9.0	$44.0 \pm 13.6^*$	$1.7 \pm 3.3^*$	$10.5 \pm 3.6^*$
BW A4C	100	110.3 ± 14.3	82.4 ± 16.8	$2.6 \pm 4.3^*$	0^*
Nedocromil	100	104.8 ± 5.2	92.9 ± 1.3	ND	ND
Azelastine	100	0^*	$8.4 \pm 5.2^*$	$1.9 \pm 2.8^*$	$0.9 \pm 1.5^*$

Drugs were added to the PMN-platelet cooperation system at the concentrations and for the time interval indicated. PMN were then challenged with $0.5 \mu\text{M}$ FMLP, and various parameters of cell activation were measured (for details see Methods). ND: not determined. The basal release of TxB_2 and LTB_4 by resting cells was $4.2 \pm 0.2 \text{ ng ml}^{-1}$ and $4.7 \pm 12.0 \text{ pg ml}^{-1}$, and upon activation by FMLP these values were $45.8 \pm 1.5 \text{ ng ml}^{-1}$ and $555.5 \pm 212.2 \text{ pg ml}^{-1}$ respectively. Results are expressed in percentage of control values. Each value is the mean $\pm$ s.d. of 3 to 4 experiments.

1 min) was the only drug tested which prevented platelet aggregation induced by FMLP-stimulated PMN.

Concentration-dependent inhibition by azelastine of PMN-mediated platelet aggregation and 5-hydroxytryptamine

Azelastine completely inhibited platelet aggregation, 5-HT release and synthesis of LTB_4 and TxB_2 . Since the anti-lipoxygenase and anti-cyclo-oxygenase activities did not account for the inhibition of platelet aggregation by stimulated PMN (both acetoxyhydroxamic compounds were ineffective), the capacity of azelastine to exert its inhibitory effect was further investigated.

Azelastine was added at increasing concentrations (5–300 μM) to a mixed cell suspension 1 min before stimulation by $0.5 \mu\text{M}$ FMLP (Figure 1). A strong platelet activation ($37.7 \pm 5.6\%$ aggregation and $59.9 \pm 19.2\%$ 5-HT release; $n = 6$) was noted in absence of azelastine, whereas with 20 μM azelastine platelet aggregation was significantly inhibited ($P < 0.05$; $n = 4$; unpaired *t*-test) and suppressed at 70 μM . Release of 5-HT was also significantly inhibited by azelastine at 30 μM ($P < 0.05$; $n = 3$). However, inhibition never reached 100% and was less apparent for high concentrations of azelastine. Thus, in the presence of 300 μM azelastine the release was $16.2 \pm 7\%$ ($n = 4$) compared to $8.9 \pm 4\%$ ($n = 5$) with 70 μM .

Figure 1 Inhibition by azelastine of platelet activation induced by FMLP-stimulated PMN: 5×10^6 PMN per ml and 2×10^8 platelets per ml were preincubated together for 4 min with cytochalasin B ($5 \mu\text{g ml}^{-1}$) and fibrinogen (0.77 mg ml^{-1}). Different azelastine concentrations were added, and 1 min later the cells were stimulated with $0.5 \mu\text{M}$ FMLP. Aggregation (stippled columns) and 5-hydroxytryptamine release (hatched columns) are expressed as percentage of activity in comparison with controls without azelastine. Each column is the mean of 4 distinct experiments; s.d. shown by vertical bars.

Effect of azelastine on PMN and platelets used separately

In order to determine whether the target of azelastine was PMN or platelets, we studied its effects on both cells separately. First, we verified that inhibition was not restricted to FMLP, and accordingly was not accounted for by receptor antagonism. Indeed, platelet activation induced by C5a-stimulated PMN was also inhibited by azelastine (aggregation: $43.2 \pm 2.1\%$ vs $3.1 \pm 0.3\%$ with 100 μM azelastine; 5-HT release: $58.4 \pm 6.5\%$ vs $0.9 \pm 0.6\%$ with 100 μM azelastine; $n = 3$).

As illustrated in Figure 2, azelastine inhibited concentration-dependently the β -glucuronidase release from FMLP-activated PMN. The effective concentrations were 20–150 μM ($P < 0.05$). However, at concentrations above 100 μM , the enzyme release was increased.

Results presented in Figure 3 show that azelastine also inhibited platelet activation, irrespective of the agonist. Indeed, platelet aggregation and 5-HT release induced by PAF (Figure 3a), or by Cat G (Figure 3b), were suppressed in a concentration-dependent manner by azelastine. Other platelet agonists such as collagen or thrombin were also inhibited by azelastine, inhibition being surmountable by increasing the concentrations of the stimulating agents (data not shown). As observed for platelet aggregation, high concentrations of azelastine never totally suppressed 5-HT release, and indeed significant amounts of 5-HT were found in supernatants of

Figure 2 Interference of azelastine with β -glucuronidase release from PMN. 5×10^6 PMN per ml were preincubated at 37°C in the presence of cytochalasin B for 4 min before addition of different azelastine concentrations. PMN were stimulated 1 min later by $0.5 \mu\text{M}$ FMLP. The amounts of enzyme released were determined in supernatants and are expressed as a percentage of total enzyme recovered in the corresponding pellets treated with Triton X-100. Each column is the mean of 4 experiments; s.d. shown by vertical bars.

Figure 3 Inhibition by azelastine of platelet aggregation induced by PAF or cathepsin G. Platelets 2×10^8 per ml of HBSS-Tyrode (1:1) were incubated with fibrinogen at 37°C. Azelastine (A) was added, and 1 min later cells were stimulated either by 1 μM of PAF (a) or by 166 nM of Cat G (b). Values in parentheses represent the percentage of 5-HT released. Tracings are representative of 4 experiments.

platelets which had not been stimulated but only incubated for 1 min with 70 μM or more azelastine. Thus, with 300 μM of the drug, the release was of $19.6 \pm 9.1\%$ ($n = 3$), which was comparable to the value observed in the PMN-platelets cooperation system (see Figure 1).

Effect of high azelastine concentrations on LDH release from PMN or platelets

Since it was likely that the effects observed for concentrations of azelastine above 100 μM (β -glucuronidase release from PMN, 5-HT release from platelets) resulted from cell permeation, lactic dehydrogenase (LDH) release was evaluated. LDH was indeed found in supernatants of PMN and platelets treated with high concentrations of azelastine, PMN being

Figure 4 Inhibition by azelastine of leukotriene B₄ (LTB₄) production in the PMN-platelet cooperation model. LTB₄ production was evaluated by radioimmunoassay from supernatants of a mixed cell suspension (PMN and platelets) pretreated with different azelastine concentrations and then stimulated with 0.5 μM FMLP. Each column is the mean of 3 experiments; vertical bars show s.d.

Figure 5 Inhibition by azelastine of thromboxane B₂ (TxB₂) production. (a) For details see Figure 4. (b) TxB₂ production was measured from supernatants of platelets pretreated with azelastine and stimulated with 166 nM of purified Cat G. Each column is the mean of 3 experiments; s.d. shown by vertical bars.

more affected than platelets since their treatment for 1 min with 100 μM azelastine induced a significant release of LDH ($P < 0.05$) whereas 200 μM were required to induce a similar effect on platelets. At 300 μM azelastine provoked a release of LDH from PMN which amounted to $63.5 \pm 13.5\%$ ($n = 3$). The same effect, although less pronounced, was observed with platelets: $36.6 \pm 13.5\%$ of LDH ($n = 3$).

Inhibition by azelastine of leukotriene B₄ and thromboxane B₂ production

Challenge of the mixed cell population with 0.5 μM FMLP led to the formation of LTB₄ (from PMN) and TxB₂ (from platelets) as shown previously (Renesto *et al.*, 1990). The results presented in Figure 4 show that LTB₄ production was strongly inhibited by azelastine. Thus, 10 μM azelastine reduced it to $2.2 \pm 2.2\%$ of the control values ($n = 3$). In addition, TxB₂ measurements performed in parallel showed an inhibition concentration-response curve (Figure 5a). The latter inhibition was a direct effect of azelastine on platelets since TxB₂ production from these cells stimulated by a submaximal concentration of Cat G was also effectively inhibited (Figure 5b).

Discussion

We have described here the effect of various drugs on PMN-mediated platelet activation. Such cell-to-cell communication, which involves the release from FMLP- or C5a-stimulated PMN of Cat G, a serine proteinase responsible for the subsequent platelet activation (Chignard *et al.*, 1986; Selak *et al.*, 1988; Renesto *et al.*, 1990; Ferrer-Lopez *et al.*, 1990; Del Maschio *et al.*, 1990) may be involved in different inflammatory processes, as mentioned in the introduction.

Among the substances tested, azelastine was the only one to inhibit PMN-induced platelet aggregation. In contrast, nedoc-

romil sodium, another anti-asthmatic drug (Eady, 1986) was inactive. ASA, ketoprofen, diclofenac and piroxicam, different anti-inflammatory substances with anti-cyclo-oxygenase activity, used at concentrations which suppressed TxB_2 formation by platelets, also failed to inhibit platelet aggregation. Furthermore, the two acetohydroxamic acids, BW 755C and BW A4C which have more anti-lipoxygenase than anti-cyclo-oxygenase properties (Randall *et al.*, 1980; Tateson *et al.*, 1988), did not affect platelet aggregation by more than 10% under conditions where LTB_4 and also TxB_2 synthesis were suppressed. This inhibition of TxB_2 formation resulted from the use of high concentrations ($100\text{ }\mu\text{M}$), of both drugs, even though BW A4C is described as a selective inhibitor of the 5-lipoxygenase activity (Tateson *et al.*, 1988). As indicated in Table 1, azelastine at $100\text{ }\mu\text{M}$ also inhibited the production of TxB_2 and LTB_4 , but the use of cyclo-oxygenase and lipoxygenase inhibitors indicates clearly that these properties do not account for the inhibition of platelet aggregation and 5-HT release.

Inhibition of platelet activation by activated PMN may result from interference (i) with PMN, (ii) with platelets, or (iii) with Cat G, the mediator involved (Renesto *et al.*, 1990; Ferrer-Lopez *et al.*, 1990). Experiments with PMN or platelet suspensions used separately, allowed us to identify the target(s) of azelastine. PMN degranulation evaluated by β -glucuronidase release was affected, the lowest effective concentration of azelastine on PMN being the same as the lowest concentration effective in the PMN-platelet suspension system, i.e., $20\text{ }\mu\text{M}$. Inhibition was not due to an antagonism at the FMLP receptor level, since activation of PMN by C5a was also prevented by azelastine. In fact, β -glucuronidase and Cat G are stored in the same azurophilic granules (Ford Bainton, 1988) and their release upon activation proceed in parallel (Renesto & Chignard, 1991 and unpublished data) on the one hand, and the degree of β -glucuronidase release is correlated with the level of platelet activation (Ferrer-Lopez *et al.*, 1990) on the other hand. Thus, it can be inferred that β -glucuronidase and Cat G release are equally affected by azelastine and that inhibition of the serine proteinase release accounts for the inhibition of platelet activation. The most effective concentration of azelastine was $50\text{ }\mu\text{M}$ but, surprisingly, higher concentrations were less potent and $300\text{ }\mu\text{M}$ did not reduce but rather enhanced β -glucuronidase release, suggesting that the drug was cytotoxic and induced PMN permeation. LDH measurements performed on the supernatants of PMN treated by this drug confirmed this hypothesis since β -glucuronidase release was always accompanied by LDH release when high concentrations of azelastine were used.

Azelastine also affected platelets since their aggregation by PAF, Cat G (Figures 3a and 3b), collagen or thrombin (not shown) was inhibited. However, unlike platelet aggregation, release of 5-HT was never totally blocked, as observed in the PMN-platelet suspension system. The fact that concentrations of azelastine above $70\text{ }\mu\text{M}$ provoked a less pronounced inhibition of 5-HT release from platelets strongly suggested a cytotoxic effect and indeed, as observed with PMN, addition of azelastine at high concentrations to platelets induced LDH release.

The evaluation of LTB_4 and TxB_2 production confirmed that the target of azelastine was not restricted to PMN but also involved platelets. Indeed, LTB_4 production from PMN

was inhibited in the cell-to-cell communication system (Figure 4). In addition, this confirmed that inhibition of LTB_4 synthesis and of platelet aggregation were not correlated, since $10\text{ }\mu\text{M}$ azelastine suppressed the former but had no effect on the latter, as observed with BW 755C and BW A4C. In these experimental conditions, TxB_2 formation was not affected by $10\text{ }\mu\text{M}$ azelastine. This means that at this concentration, azelastine does not interfere with the platelet function (as confirmed with exogenously added Cat G, Figures 3b and 5b), nor with PMN stimulation, as evaluated by the inhibition of β -glucuronidase release (Figure 2), but is fully active against LTB_4 formation. This indicates a specific 5-lipoxygenase inhibitory activity, as shown by Nishihira *et al.* (1989). At concentrations above $70\text{ }\mu\text{M}$, azelastine inhibited TxB_2 formation by Cat G-activated platelets dose-dependently (Figure 5b). This probably results from an effect unrelated to cyclo-oxygenase and/or thromboxane synthase inhibition, since aggregation was similarly affected by azelastine whereas the tested anti-inflammatory drugs with anti-cyclo-oxygenase properties suppressed TxB_2 formation without modifying aggregation.

The mechanism by which azelastine interferes with allergy is not established. It has been shown that it prevents the synthesis/release of mediators such as histamine (Chand *et al.*, 1985), leukotrienes (Chand *et al.*, 1989; Nishihira *et al.*, 1989), superoxide anions (Busse *et al.*, 1989; Taniguchi & Takanaka, 1989), arachidonic acid (Taniguchi & Takanaka, 1989), and that it antagonizes the effect of some of these mediators on target organs (Rafferty *et al.*, 1989). In addition, Nakamura *et al.* (1988) have shown that azelastine inhibits the release of Ca^{2+} from intracellular storage sites in guinea-pig peritoneal macrophages stimulated by PAF or by FMLP. Azelastine was also described as an inhibitor of leukocyte (Chand *et al.*, 1985; Taniguchi & Takanaka, 1989; Busse *et al.*, 1989), and platelet functions (Achterrath-Tuckermann *et al.*, 1988). Azelastine is effective in animals (Chand *et al.*, 1986; 1987; Achterrath-Tuckermann *et al.*, 1988), and has been claimed to reduce allergic rhinitis (Meltzer *et al.*, 1988) and asthma (Spector *et al.*, 1987; Kemp *et al.*, 1987). The present paper supports a specific target for this drug, i.e., the ability to inhibit PMN-mediated platelet activation, a property which is shared neither by cyclo-oxygenase and lipoxygenase inhibitors nor by nedocromil sodium. Interestingly, experiments performed on isolated lungs from sensitized guinea-pigs demonstrated that nedocromil sodium inhibits the establishment of hyperresponsiveness but is unable to prevent its manifestation once established (Pretolani *et al.*, 1990). By contrast and in parallel with the present results, azelastine is capable of inhibiting these same manifestations of hyperresponsiveness. In other words, azelastine suppressed the activation of inflammatory cells present in the lungs of sensitized animals (Pretolani *et al.*, unpublished data).

In the present study we have shown that azelastine interferes with the cell-to-cell communication between human PMN and platelets. This effect may be relevant for its therapeutic efficiency in asthma and may also suggest therapeutic values in pathologies in which PMN and platelets are involved.

The authors thank Dr A.J. Coyle for his advice in the preparation of the manuscript, and Laboratories Sarget for generous support.

References

- ACHTERRATH-TUCKERMANN, U., WEISCHER, C.H. & SZELENYI, I. (1988). Azelastine, a new antiallergic/asthmatic agent, inhibits PAF-acether-induced-platelet aggregation, paw edema and bronchoconstriction. *Pharmacology*, **36**, 265–271.
- BAGGIOLINI, M. & DEWALD, B. (1985). The neutrophil. *Int. Arch. Allergy Appl. Immunol.*, **76** (suppl. 1), 13–20.
- BAUGH, R.J. & TRAVIS, J. (1976). Human leukocyte granule elastase: rapid isolation and characterization. *Biochemistry*, **15**, 836–841.
- BEDNAR, M., SMITH, B., PINTO, A. & MULLANE, K.M. (1985). Neutrophil depletion suppress ^{111}In -labeled platelet accumulation in infarcted myocardium. *J. Cardiovasc. Pharmacol.*, **7**, 906–912.
- BUSSE, W., RANDLEV, B. & SEDGWICK, J. (1989). The effect of azelastine on neutrophil and eosinophil generation of superoxide. *J. Allergy Clin. Immunol.*, **83**, 400–405.
- CHAND, N., NOLAN, K., DIAMANTIS, W., PERHACH, J.L. & SOFIA, R.D. (1986). Inhibition of leukotriene (SRS-A)-mediated acute lung anaphylaxis by azelastine in guinea pigs. *Allergy*, **41**, 473–478.
- CHAND, N., NOLAN, K., DIAMANTIS, W. & SOFIA, R.D. (1987). Inhibi-

- tion of acute lung anaphylaxis by aerosolized azelastine in guinea-pigs sensitized by three different procedures. *Ann. Allergy*, **58**, 344–349.
- CHAND, N., PILLAR, J., DIAMANTIS, W. & SOFIA, R.D. (1985). Inhibition of allergic histamine release by azelastine and selected anti-allergic drugs from rabbit leucocytes. *Int. Arch. Allergy. Appl. Immunol.*, **77**, 451–455.
- CHAND, N., PILLAR, J., NOLAN, K., DIAMANTIS, W. & SOFIA, R.D. (1989). Inhibition of allergic and nonallergic leukotriene C₄ formation and histamine secretion by azelastine: Implication for its mechanism of action. *Int. Arch. Allergy. Appl. Immunol.*, **90**, 67–70.
- CHIGNARD, M., LE COUEDIC, J.P., TENCE, M., VARGAFTIG, B.B. & BENVENISTE, J. (1979). The role of platelet-activating factor in platelet aggregation. *Nature*, **279**, 799–800.
- CHIGNARD, M., SELAK, M.A. & SMITH, J.B. (1986). Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 8609–8613.
- DEL MASCHIO, A., EVANGELISTA, V., RAJTAR, G., MIN CHEN, Z., CERLETTI, C. & DE GAETANO, G. (1990). Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am. J. Physiol.*, **258**, H870–H879.
- DURHAM, S.R., CARROLL, M., WALSH, G.M. & KAY, A.B. (1984). Leukocyte activation in allergen-induced late-phase asthmatic reactions. *N. Engl. J. Med.*, **311**, 1398–1402.
- EADY, R.P. (1986). The pharmacology of nedocromil sodium. *Eur. J. Respir. Dis.*, **69** (suppl. 147), 112–119.
- FERRER-LOPEZ, P., RENESTO, P., SCHATTNER, M., BASSOT, S., LAURENT, P. & CHIGNARD, M. (1990). Activation of human platelets by C5a-stimulated neutrophils: a role for cathepsin G. *Am. J. Physiol.*, **258**, C1100–C1107.
- FORD BAITON, D. (1988). Phagocytic cells: developmental biology of neutrophils and eosinophils. In *Inflammation: Basic Principles and Clinical Correlates*. ed. Gallin, J.I., Goldstein, I.M. & Snyderman, R. pp. 265–277. New York: Raven Press.
- HEFFNER, J.E., SAHN, S.A. & REPINE, J.E. (1987). The role of platelets in the adult respiratory distress syndrome. Culprits or bystanders? *Am. Rev. Respir. Dis.*, **135**, 482–492.
- ISSEKUTZ, A.C., RIPLEY, M. & JACKSON, J.R. (1983). Role of neutrophils in the deposition of platelet during acute inflammation. *Lab. Invest.*, **49**, 716–724.
- JOHNSON, R.J., ALPERS, C.E., PRITZL, P., SCHULZE, M., BAKER, P., PRUCHNO, C. & COUSER, W.G. (1988). Platelets mediate neutrophil-dependent immune complex nephritis in the rat. *J. Clin. Invest.*, **82**, 1225–1235.
- KAY, A.B. (1987). Inflammatory cells in acute and chronic asthma. *Am. Rev. Respir. Dis.*, **135**, S63–S66.
- KEMP, J.P., MELTZER, E.O., ORGEL, H.A., WELCH, M.J., BUCHOLTZ, G.A., MIDDLETON, E., SPECTOR, S.L., NEWTON, J.J. & PERHACH, J.L. (1987). A dose-response study of the bronchodilator action of azelastine in asthma. *J. Allergy Clin. Immunol.*, **79**, 893–899.
- MARCUS, A.J., SAFIER, L.B., ULLMAN, H.L., ISLAM, N., BROEKMAN, M.J., FALCK, J.R., FISCHER, S. & VON SCHACKY, C. (1988). Platelet-neutrophil interactions. (12S)-hydroxyeicosatetraen-1,20-dioic acid: a new eicosanoid synthesized by unstimulated neutrophils from (12S)-20-dihydroxyeicosatetraenoic acid. *J. Biol. Chem.*, **263**, 2223–2229.
- MARTODAM, R.R., BAUGH, R.J., TWUMASI, D.Y. & LIENER, I.E. (1979). A rapid procedure for the large scale purification of elastase and cathepsin G from human sputum. *Prep. Biochem.*, **9**, 15–31.
- MELTZER, E.O., STORMS, W.W., PIERSON, W.E., CUMMINS, L.H., ORGEL, H.A., PERHACH, J.L. & HEMSWORTH, G.R. (1988). Efficacy of azelastine in perennial allergic rhinitis: Clinical and rhinomanometric evaluation. *J. Allergy Clin. Immunol.*, **82**, 447–455.
- NAKAMURA, T., NISHIZAWA, Y., SATO, T. & YAMATO, C. (1988). Effect of azelastine on the intracellular Ca²⁺ mobilization in guinea pig peritoneal macrophages. *Eur. J. Pharmacol.*, **148**, 35–41.
- NISHIHARA, J., HAYAKAWA, T., SUZUKI, K., KATO, K. & ISHIBASHI, T. (1989). Effect of azelastine on leukotriene synthesis in murine peritoneal cells and on thromboxane synthesis in human platelets. *Int. Arch. Allergy. Appl. Immunol.*, **90**, 285–290.
- PAGE, C.P. (1989). Platelets as inflammatory cells. *Immunopharmacol.*, **17**, 51–59.
- PRETOLANI, M., LEFORT, J., SILVA, P., MALANCHERE, E., DUMAREY, C., BACHELET, M. & VARGAFTIG, B.B. (1990). Protection by nedocromil sodium of active immunization-induced bronchopulmonary alterations in the guinea-pig. *Am. Rev. Respir. Dis.*, **141**, 1259–1265.
- RAFFERTY, P., PHILLIPS, W.H., CLOUGH, J., CHURCH, M.K., AURICH, R., OLLIER, S. & HOLGATE, S.T. (1989). The inhibitory actions of azelastine hydrochloride on the early and late bronchoconstrictor responses to inhaled allergen in atopic asthma. *J. Allergy Clin. Immunol.*, **84**, 649–657.
- RANDALL, R.W., EAKINS, K.E., HIGGS, G.A., SALMON, J.A. & TATESON, J.E. (1980). Inhibition of arachidonic acid cyclooxygenase and lipoxygenase activities of leukocytes by indomethacin and compound BW 755C. *Agents Actions*, **10**, 553–555.
- RENESTO, P. & CHIGNARD, M. (1991). Tumor necrosis factor alpha enhances platelet activation via cathepsin G released from neutrophils. *J. Immunol.*, (in press).
- RENESTO, P., FERRER-LOPEZ, P. & CHIGNARD, M. (1990). Interference of recombinant eglin C, a proteinase inhibitor extracted from leeches, with neutrophil-mediated platelet activation. *Lab. Invest.*, **62**, 409–416.
- SELAKE, M.A., CHIGNARD, M. & SMITH, J.B. (1988). Cathepsin G is a strong platelet agonist released by neutrophils. *Biochem. J.*, **251**, 293–299.
- SPECTOR, S.L., PERHACH, J.L., ROHR, A.S., RACHELEFSKY, G.S., KATZ, R.M. & SIEGEL, S.C. (1987). Pharmacodynamic evaluation of azelastine in subjects with asthma. *J. Allergy. Clin. Immunol.*, **80**, 75–80.
- TANIGUCHI, K. & TAKANAKA, K. (1989). Inhibitions of metabolic responses of polymorphonuclear leukocytes by anti-allergic drugs. *J. Pharmacobio.-Dyn.*, **12**, 37–42.
- TATESON, J.E., RANDALL, R.W., REYNOLDS, C.H., JACKSON, W.P., BHATTACHERJEE, P., SALMON, J.A. & GARLAND, L.G. (1988). Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: biochemical assessment *in vitro* and *ex vivo*. *Br. J. Pharmacol.*, **94**, 528–539.
- TVEDTEN, H.W., TILL, G.O. & WARD, P.A. (1985). Mediators of lung injury in mice following systemic activation of complement. *Am. J. Pathol.*, **119**, 92–100.
- WARSHAWSKI, F.J., SIBBALD, W.J., DRIEDGER, A.A. & CHEUNG, H. (1986). Abnormal neutrophil-pulmonary interaction in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.*, **133**, 797–804.
- WEISS, S.J. (1989). Tissue destruction by neutrophils. *N. Engl. J. Med.*, **320**, 365–376.

(Received December 14, 1990
 Revised January 9, 1991
 Accepted February 12, 1991)