

Differential expression of platelet activation markers in aspirin-sensitive asthmatics and normal subjects

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Summary

Background Activation of platelets and expression of adhesion molecules (e.g. CD62P and CD63) which mediate interactions between platelets and other cells may be important in the pathogenesis of aspirin-sensitive asthma.

Objective To determine the expression of CD62P and CD63 on platelets from aspirin-sensitive asthmatic (ASA+), aspirin-tolerant asthmatic (ASA–) and normal subjects and to assess the modulatory effect of aspirin on platelet CD62P and CD63 expression following stimulation with either platelet-activating factor (PAF), arachidonic acid (AA) or collagen (COL).

Methods Platelet-rich plasma was obtained from 10 ASA+, 10 ASA– and 10 normal control subjects, and expression of CD62P and CD63 was measured by flow cytometry. Platelets were stimulated with PAF (10, 80 nM), AA (0.1, 1 mM) or COL (80, 800 µg/mL) with or without aspirin (concentration range 0.4–4 mg/mL).

Results In the absence of aspirin, CD62P expression induced by AA and COL was greater in ASA+ patients compared with control subjects ($P < 0.001$) while CD62P expression with PAF, AA and COL was reduced in ASA– when compared with ASA+ and control subjects ($P < 0.001$). CD63 expression with PAF and AA was reduced in both ASA+ and ASA– patients compared with control subjects ($P < 0.001$). Aspirin inhibited the expression of both CD62P and CD63 after agonist stimulation. Greater inhibition of CD62P expression was observed in ASA+ compared with ASA– patients ($P < 0.001$) and normal subjects ($P < 0.05$) while greater inhibition of CD63 expression was observed in normal subjects compared with both ASA+ and ASA– patients ($P < 0.05$). In ASA+ patients and normal subjects, stimulation with PAF and COL resulted in only one platelet population while in contrast with 1 mM AA two populations were observed.

Conclusions Enhanced AA– and collagen-induced platelet CD62P expression in ASA+ patients compared with normal subjects and greater inhibition by aspirin of CD62P expression in ASA+ may be relevant to the pathogenesis of this syndrome. Reduced expression of CD62P and CD63 in platelets of ASA– patients following stimulation with PAF and AA may also have implications for the role of platelets and these mediators in the pathogenesis of other forms of asthma.

Keywords: platelets, aspirin-sensitive asthma, CD62P, CD63, platelet-activating factor, arachidonic acid, collagen

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Introduction

Platelets are well recognized for their involvement in

thrombosis and haemostasis but increasing evidence suggests they also play an important role in respiratory disorders such as asthma. Platelets are present on the epithelial surface of lung tissue from symptomatic asthmatics [1], within the microvasculature of lung tissue [2] and in bronchoalveolar lavage (BAL) fluid from asthmatic patients following allergen challenge [3]. Low affinity IgE receptors on platelets can be activated during IgE-dependent events [4] and a number of studies have suggested that platelet morphology and function may be abnormal in atopic asthma (reviewed in [5]). Thus, platelet survival time is shortened [6] while bleeding time and platelet mass may be increased [7].

In symptomatic asthma, nocturnal asthma and following allergen-induced bronchoconstriction, plasma concentrations of platelet factor 4 and β -thromboglobulin have been reported to increase in some but not all studies [5,8–11] and release of these proteins correlates with increases in albumin, eosinophil granule proteins and prostanoids in BAL fluid from asthmatics challenged with allergen [12]. A specific abnormality of platelet function may also occur in aspirin-sensitive asthma (ASA+), as platelets from ASA+ patients but not from aspirin-tolerant (ASA–) patients or normal subjects generate oxygen free radicals when exposed to aspirin or non-steroidal anti-inflammatory drugs *in vitro* [13]. Although this finding has not been confirmed in other studies [14,15], the activity of the enzyme glutathione peroxidase that metabolises hydrogen peroxide, is reduced in the platelets of ASA+ patients [14,16].

While these findings suggest the probable involvement of activated platelets in the pathogenesis of atopic and ASA+, the precise mechanisms have yet to be determined. The recent development of monoclonal antibodies to platelet activation specific antigens has facilitated the use of flow cytometry as a more sensitive technique for measuring platelet activation compared with aggregometry or assays of secreted products [17–20]. Activation of platelets causes degranulation and fusion of the granule and plasma membranes with translocation of activation dependent antigens to the external surface where they function as adhesive proteins [17,18,20].

CD62P (P-selectin, GMP-140, PADGEM), a 140 kDa glycoprotein which is preformed and stored for rapid release in the α granules of platelets [21,22] potentially facilitates the interaction of activated platelets with neutrophils and monocytes [23,24]. CD63 is a 53 kDa lysosomal integrin membrane glycoprotein expressed on platelets, monocytes and macrophages [25,26], and may also be weakly expressed on T cells, B cells and granulocytes [27]. A definitive biological function for CD63 is not known but its broad cellular distribution suggests

that it may participate in the signal transduction processes between platelets and other cells.

Differential expression of CD62P and CD63 has been observed following stimulation of platelets from normal subjects [28] but information on platelet CD62P and CD63 expression in various disease states is limited [29,30]. Moreover, *in vitro* comparisons of platelet aggregation in atopic asthmatic and normal subjects have produced conflicting results [5], although differences in platelet aggregation and ATP release in ASA+ patients compared with normal subjects have been observed [31]. In order to extend these studies and further explore the possible role of platelets in the pathology of ASA+, the present study has compared the effect of aspirin in combination with the physiologically relevant agonists, platelet-activating factor (PAF), arachidonic acid (AA) and collagen (COL), on platelet CD62P and CD63 expression in ASA+ and ASA– patients and normal subjects.

Materials and methods

Subjects

The study population comprised 10 stable ASA+ patients, 10 stable ASA– patients and 10 normal volunteers. The latter two groups were age- and sex-matched with the ASA+ patients (five women and five men). The mean ($\pm$ SD) ages were 56.9 ± 7.2 years for ASA+ patients, 51.2 ± 5.4 years for ASA– patients and 47.1 ± 7.5 years for normal subjects. The ASA+ patients were selected on the basis of a past clinical history of at least two documented episodes of acute asthmatic responses to oral aspirin and/or a positive response to a formal aspirin challenge. In addition, these patients had many of the described clinical features associated with this syndrome namely, eosinophilia, rhinitis and sinusitis and nasal polyposis while seven of these patients were atopic. The ASA– patients were all atopic and had no history of sensitivity to aspirin or NSAID. All of the asthma patients were receiving as required, inhaled β_2 -adrenoceptor agonists and 15 (eight ASA+ patients and seven ASA– patients) were taking inhaled corticosteroids. No other asthma medications were being used. Asthma had been stable in all patients for at least 4 weeks prior to the study. All subjects were asked to abstain from taking aspirin or NSAID for at least 4 weeks prior to entering the study.

Materials

Agonists Calf skin collagen (COL, Sigma Chemical Co. Ltd, St Louis, MO, USA) and arachidonic acid (AA,

Biodata Corporation, Horsham, PA, USA) were dissolved in distilled water. Stock solutions of phorbol myristate acetate (PMA, Sigma Chemical Co. Ltd) in dimethyl sulphoxide and platelet-activating factor (PAF, Sigma Chemical Co. Ltd) in chloroform-methanol (1 : 1) were diluted to required concentrations in phosphate buffered saline containing 1% (w/v) bovine serum albumin (PBS/BSA) on the day of each experiment.

Monoclonal antibodies Phycoerythrin labelled anti-CD62P (CD62/PE, 1.5 µg/mL; Becton Dickinson, Mountainview, CA, USA), fluorescein isothiocyanate labelled anti-CD63 (CD63/FITC, Immunotech, Marseille, France) and mouse isotype control IgG1FITC/IgG2aPE antibodies (Becton Dickinson) were diluted in PBS/BSA prior to use.

Methods

Platelet preparation To minimize platelet activation, blood was collected atraumatically from an antecubital vein through a 19-gauge butterfly needle. The first 4 mL of blood were discarded and the subsequent 9 mL were collected in a Vacutainer tube (Becton Dickinson) containing 1 mL of acid-citrate and dextrose. Platelet rich plasma (PRP) was obtained by centrifugation at $150 \times g$ for 15 min. The platelet preparation was diluted to 10^7 cells/mL in PBS/BSA.

Assessment of platelet count and mean platelet volume

Whole blood samples were diluted 1 : 50 000 in Isoton (Coulter Electronics Pty Ltd, Hialeah, Florida, USA) and platelet counts and mean platelet volume were measured in triplicate on a PLT4 platelet counter (SEAC, Firenze, Italy). Platelet mass was calculated as the product of platelet count and mean platelet volume [7].

Assay of platelet adhesion receptor expression by flow cytometry

Aliquots of PRP (50 µL) were activated with submaximal and maximal concentrations of PAF (10, 80 nM), AA (0.1, 1 mM) and COL (80, 800 µg/mL) as assessed from previous studies on platelet aggregation [32] and CD62P and CD63 expression [28]. PMA (final concentration, 1.6 µM) was used as a positive control to determine the maximum possible platelet activation marker expression in each sample. After incubation for 5 min at room temperature, 10 µL of anti-CD63/FITC or

anti-CD62P/PE (both diluted 1 : 25 with PBS/BSA) were added to each tube. The negative control comprised 10 µL of mouse isotype IgG1FITC/IgG2aPE. The samples were then incubated in the dark, at room temperature, for 30 min and the platelets were then fixed with 1% (v/v) formaldehyde and stored at 4°C until analysed in a Coulter Epics Profile II flow cytometer. The cytometer used a 20 mW Argon laser operated at 15 mW power and 488 nm. Fluorescein fluorescence was detected at 520 nm whereas phycoerythrin fluorescence was detected at 576 nm. The instrument was calibrated daily for fluorescence and light scatter using Immuno beads (Coulter Electronics Pty Ltd). Samples were passed through a 75 µm nozzle at a flow rate of 15 µL per min. Analytical gates were set around the platelet population using forward angle light scatter, and all data were collected in log mode using single parameter analysis. Debris or 'machine noise' demonstrated a scatter profile distinctly smaller than that of platelets and was excluded from the analysis by setting the appropriate forward-scatter threshold. A gate was set around the platelets, and 5000 cells were analysed to quantitate the number of platelets binding antibody which was then expressed as the percent positive platelets detected.

Modulation of CD62P and CD63 expression by aspirin

Aliquots of PRP (50 µL) were incubated with varying concentrations of aspirin (0, 0.4, 0.8, 2, 4 mg/mL) for 20 min. Buffer (unstimulated) or varying concentrations of agonists (PAF, AA, COL) were then added and the expression of CD62P and CD63 was measured by flow cytometry.

Analysis and statistics The degree of inhibition of marker expression was determined by expressing the value obtained post-aspirin exposure as a percentage of the response obtained without aspirin. The % inhibition was plotted against the aspirin concentration and the area under this curve (AUC) determined for each subject using the trapezoidal rule (Fig P, FigP Software Corporation, North Carolina, USA). From the same curve, the concentration of aspirin producing 50% inhibition of the maximum control value (IC_{50}) was determined for each subject for each agonist. Simple descriptive statistics were used to generate mean ($\pm$ SEM) data while statistical comparisons were performed using Student's *t*-test, ANOVA and Tukey-Kramer's multiple

Fig. 1. Histograms representing CD62P expression induced by (a) and (b) platelet activating factor (PAF, 10 and 80 nM), (c) and (d) arachidonic acid (AA, 0.1 and 1 mM) and (e) and (f) collagen (COL, 80 and 800 µg/mL). The values are means ($\pm$ SEM) from 10 aspirin-sensitive asthmatics (ASA+), 10 aspirin-tolerant asthmatics (ASA-) and 10 normal subjects.

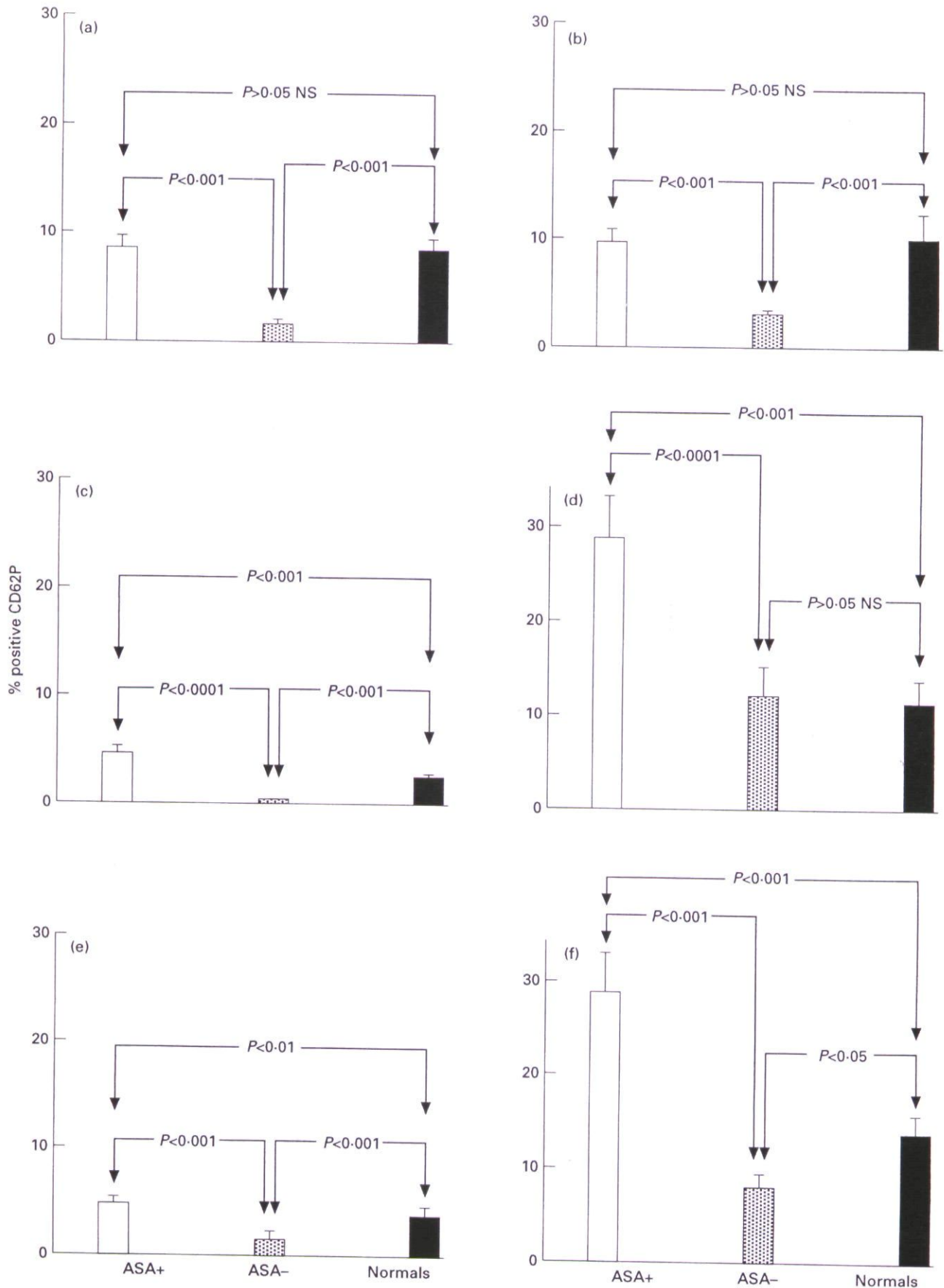


Table 1. Activation antigen expression (mean $\pm$ SEM) on unstimulated platelets from ASA+, ASA– and Normal subjects

	Before aspirin		After aspirin	
	CD62P	CD63	CD62P	CD63
ASA+ ($n = 10$)	1.1 $\pm$ 0.6	1.95 $\pm$ 0.8	1.6 $\pm$ 0.7	2.1 $\pm$ 1.1
ASA– ($n = 10$)	0.9 $\pm$ 0.2	2.02 $\pm$ 0.7	1.4 $\pm$ 0.5	1.9 $\pm$ 0.8
Normals ($n = 10$)	0.8 $\pm$ 0.07	1.98 $\pm$ 1	0.9 $\pm$ 0.1	2.3 $\pm$ 1.2

($P > 0.5$ for both antigens).

comparison test using the statistical program Instat (Graph Pad, San Diego, USA).

Results

Mean platelet counts, platelet volume and platelet mass

The mean whole blood platelet count was significantly higher in ASA+ patients ($525 \pm 8.4 \times 10^9/L$) and ASA– patients ($506 \pm 15 \times 10^9/L$) compared with normal subjects ($452 \pm 20 \times 10^9/L$; $P = 0.006$, ANOVA). Mean whole blood platelet volume was higher in ASA+ (8.1 ± 1.5 fl) and ASA– patients (7.86 ± 0.8 fl) than in normal subjects (7.06 ± 1.2 fl), but the differences were not statistically significant. However, the platelet mass was significantly higher in ASA+ (5.19 ± 0.12 mL/L) and ASA– patients (4.85 ± 0.1 mL/L) compared with normal subjects (3.89 ± 0.1 mL/L; $P < 0.0001$, ANOVA).

CD62P and CD63 expression prior to aspirin exposure

There was significantly greater expression of CD62P on platelets from ASA+ patients compared with platelets from normal subjects when induced by submaximal and maximal concentrations of AA (0.1 and 1 mM) and COL (80 and 800 μ g/mL) (Fig. 1). In contrast, there was significantly reduced expression of CD63 in ASA+ patients compared with normal subjects when induced by PAF (10 and 80 nM) and AA (0.1 and 1 mM) but not COL (Fig. 2). Platelets from ASA– patients expressed significantly less CD62P and CD63 compared with ASA+ patients and normal subjects in response to PAF, AA and COL at both submaximal and maximal concentrations of the agonists (Figs 1 and 2). Mean

PMA-induced expression of CD62P in ASA+ patients, ASA– patients and normal subjects was 70.7 ± 7.7 , 69.6 ± 4.5 and $72.9 \pm 5.2\%$ respectively, while mean CD63 expression was 73.5 ± 6.8 , 74.1 ± 7.1 and $70.3 \pm 5.8\%$ respectively.

Linear regression analysis of the effects of different agonists on the expression of CD62P in normal subjects demonstrated a positive correlation between the maximum per cent platelet expression induced by PAF and AA ($r = 0.74$, $P = 0.013$). Similarly with CD63, a positive correlation was obtained between PAF and AA ($r = 0.63$, $P = 0.05$). No other significant correlations were observed. In ASA+ and ASA– patients, no significant correlations were observed for either CD62P or CD63.

Effect of aspirin alone on CD62P and CD63 expression

In all three groups of subjects aspirin alone had no significant effect on the expression of CD62P and CD63 ($P > 0.5$ for both antigens, Table 1).

Effect of aspirin on CD62P and CD63 expression induced by PAF, AA and COL

Analysis of the areas under the curve (AUC) of % inhibition by aspirin indicated significantly greater inhibition of CD62P expression in ASA+ patients compared with normal subjects for PAF (10 and 80 nM), AA (0.1 and 1 mM) and COL (800 μ g/mL, Fig. 3). In contrast, greater inhibition of CD63 expression occurred in normal subjects compared with ASA+ patients for PAF (10 and 80 nM), AA (0.1 mM) and COL (80 μ g/mL, Fig. 4). Compared with normal subjects and ASA+

Fig. 2. Histograms representing CD63 expression induced by (a) and (b) platelet activating factor (PAF, 10 and 80 nM), (c) and (d) arachidonic acid (AA, 0.1 and 1 mM) and (e) and (f) collagen (COL, 80 and 800 μ g/mL). The values are means ($\pm$ SEM) from 10 aspirin-sensitive asthmatics (ASA+), 10 aspirin-tolerant asthmatics (ASA–) and 10 normal subjects.

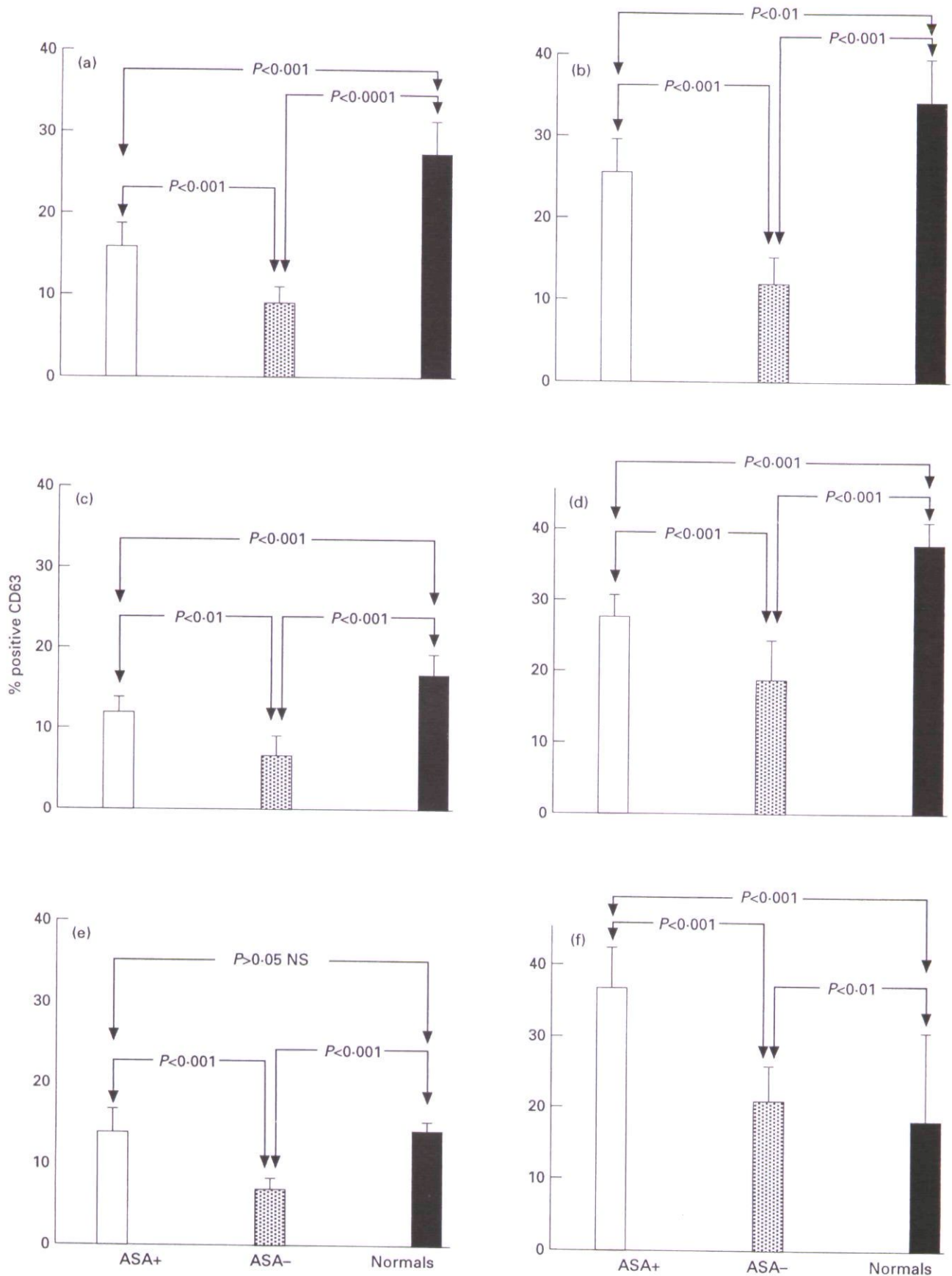


Table 2. The inhibitory effect of aspirin on CD62P expression induced by PAF, AA and COL

Agonist (concentration)	ASA+ IC ₅₀ (mg)	ASA- IC ₅₀ (mg)	Normal IC ₅₀ (mg)	P-value (ANOVA)	Tukey-Kramer (multiple comparison test)
PAF (10 nM)	0.40 ± 0.06	1.05 ± 0.3	0.62 ± 0.08	0.001	ASA+ vs ASA- <i>P</i> < 0.001 ASA+ vs normals <i>P</i> < 0.05 ASA- vs normals <i>P</i> < 0.01
PAF (80 nM)	0.20 ± 0.03	0.62 ± 0.09	0.48 ± 0.07	0.0001	ASA+ vs ASA- <i>P</i> < 0.001 ASA+ vs normals <i>P</i> < 0.001 ASA- vs normals <i>P</i> < 0.05
AA (0.1 mM)	0.4 ± 0.07	1.2 ± 0.1	0.8 ± 0.4	0.0001	ASA+ vs ASA- <i>P</i> < 0.0001 ASA+ vs normals <i>P</i> < 0.001 ASA- vs normals <i>P</i> < 0.01
AA (1 mM)	0.59 ± 0.09	1 ± 0.2	0.74 ± 0.05	0.0001	ASA+ vs ASA- <i>P</i> < 0.001 ASA+ vs normals <i>P</i> < 0.001 ASA- vs normals <i>P</i> < 0.001
COL (80 µg/mL)	1.5 ± 0.2	2.9 ± 0.86	1.3 ± 0.4	0.001	ASA+ vs ASA- <i>P</i> < 0.001 ASA+ vs normals ns ASA- vs normals <i>P</i> < 0.001
COL (800 µg/mL)	0.24 ± 0.06	2.35 ± 0.94	0.47 ± 0.03	0.0001	ASA+ vs ASA- <i>P</i> < 0.0001 ASA+ vs normals ns ASA- vs normals <i>P</i> < 0.0001

Values represent means ± SEM of the 50% inhibitory concentration IC₅₀ data (*n* = 10). *P*-values indicate that means were significantly different between the aspirin-sensitive asthmatics (ASA+), aspirin-tolerant asthmatics (ASA-) and normal subjects.

patients, CD62P expression in platelets from ASA- patients was the least inhibited by aspirin when PAF (10 and 80 nM) and COL (800 µg/mL) were used as agonists (Fig. 3). Similarly, CD63 expression in platelets from ASA- patients was the least inhibited by aspirin when PAF (10 and 80 nM) and AA (0.1 and 1 mM) were used as agonists (Fig. 4).

Similar results to those seen in the AUC analysis were obtained when the concentrations of aspirin producing 50% inhibition of the maximum control value (IC₅₀) were calculated. The aspirin IC₅₀ for CD62P expression induced by PAF (10 and 80 nM), AA (0.1 and 1 mM) and COL (800 µg/mL) was significantly lower in ASA+ patients compared with ASA- patients and normal subjects (Table 2). In contrast, for PAF (10 and 80 nM), AA (0.1 and 1 mM) and COL (80 µg/mL), a significantly lower aspirin IC₅₀ was obtained for CD63 expression on platelets from normal subjects compared with platelets from ASA+ and ASA- patients. For expression of both CD62P and CD63 induced by each of the agonists, platelets from ASA- patients demonstrated a

higher IC₅₀ for inhibition by aspirin compared with platelets from ASA+ patients and normal subjects (Tables 2 and 3).

Linear regression analysis of the inhibitory effects of aspirin on PAF, AA and COL induced CD62P and CD63 expression in normal subjects demonstrated a significant correlation between % inhibition for PAF and COL for CD62P alone (*r* = 0.62, *P* = 0.05). Although a similar trend was observed in ASA+ patients, it was not statistically significant.

Platelet subpopulations

The scatter profiles demonstrated qualitatively different effects for CD62P and CD63 expression in normal subjects in response to the various agonists. With PAF and COL, only one platelet population was observed with all agonist concentrations, while with 1 mM AA, two distinct platelet populations were observed (Fig. 5). However, only one population was observed with submaximal (0.1 mM) and supramaximal (10 mM) concentrations of

Fig. 3. Histograms representing the inhibition by aspirin of CD62P expression induced by (a) and (b) platelet activating factor (PAF, 10 and 80 nM), (c) and (d) arachidonic acid (AA, 0.1 and 1 mM) and (e) and (f) collagen (COL, 80 and 800 µg/mL). The values are mean (± SEM)% inhibition of expression obtained by area under the curve (AUC) analysis of data from 10 aspirin-sensitive asthmatics (ASA+), 10 aspirin-tolerant asthmatics (ASA-) and 10 normal subjects.

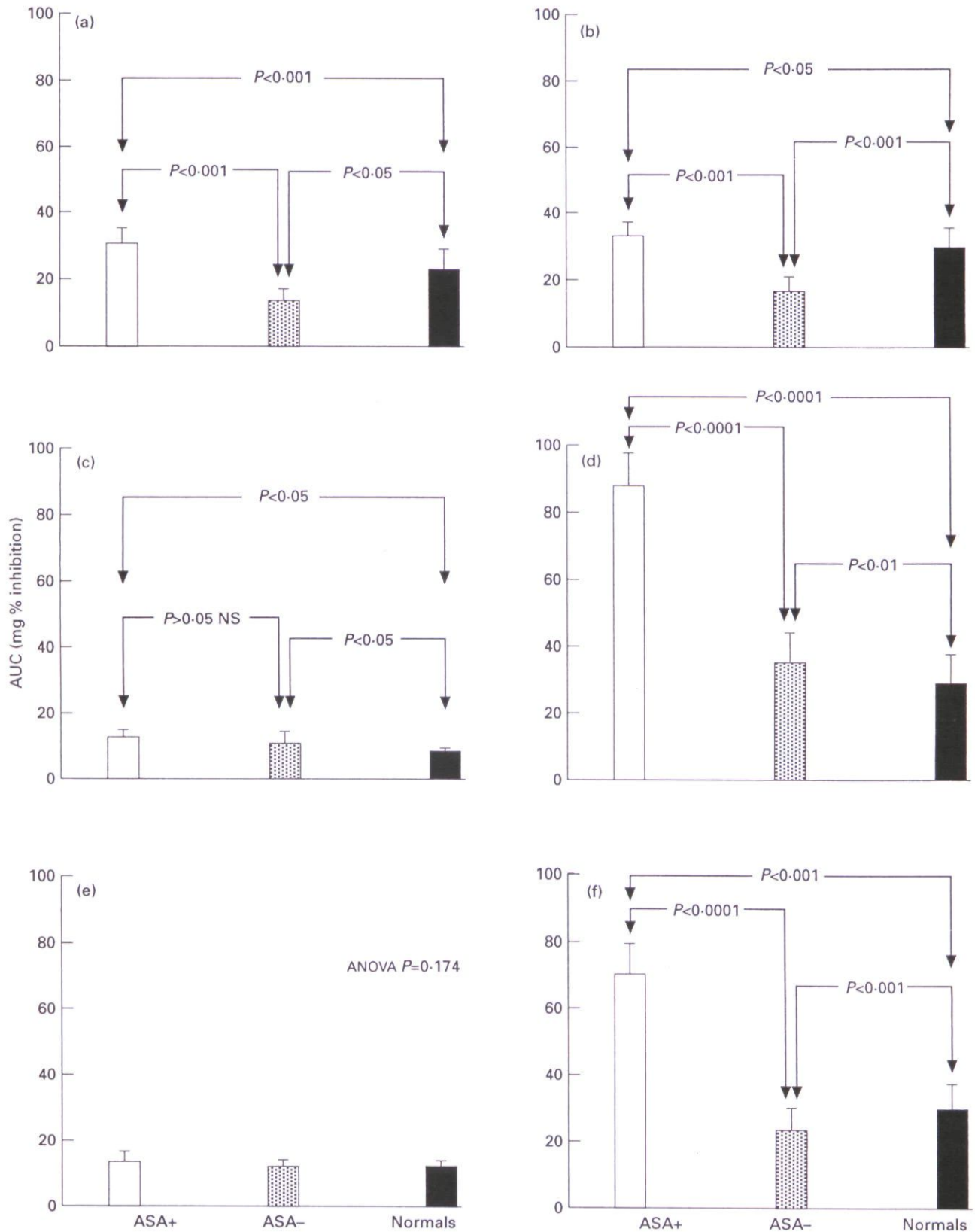


Table 3. The inhibitory effect of aspirin on CD63 expression induced by PAF, AA and COL

Agonist (concentration)	ASA+ IC ₅₀ (mg)	ASA- IC ₅₀ (mg)	Normal IC ₅₀ (mg)	P-value (ANOVA)	Tukey-Kramer (multiple comparison test)
PAF (10 nM)	0.74 ± 0.09	0.91 ± 0.08	0.32 ± 0.06	0.0001	ASA+ vs ASA- NS ASA+ vs normals <i>P</i> < 0.001 ASA- vs normals <i>P</i> < 0.001
PAF (80 nM)	0.32 ± 0.08	0.65 ± 0.09	0.20 ± 0.04	0.001	ASA+ vs ASA- <i>P</i> < 0.001 ASA+ vs normals NS ASA- vs normals <i>P</i> < 0.001
AA (0.1 mM)	0.50 ± 0.07	1.94 ± 0.9	0.34 ± 0.05	0.0001	ASA+ vs ASA- <i>P</i> < 0.0001 ASA+ vs normals NS ASA- vs normals <i>P</i> < 0.0001
AA (1 mM)	0.73 ± 0.1	1.38 ± 0.5	0.59 ± 0.08	0.0001	ASA+ vs ASA- <i>P</i> < 0.001 ASA+ vs normals <i>P</i> < 0.05 ASA- vs normals <i>P</i> < 0.001
COL (80 µg/mL)	0.65 ± 0.07	0.76 ± 0.07	0.35 ± 0.09	0.001	ASA+ vs ASA- <i>P</i> < 0.05 ASA+ vs normals <i>P</i> < 0.001 ASA- vs normals <i>P</i> < 0.001
COL (800 µg/mL)	0.50 ± 0.05	0.67 ± 0.02	0.59 ± 0.08	0.0001	ASA+ vs ASA- <i>P</i> < 0.01 ASA+ vs normals NS ASA- vs normals NS

Values represent means ± SEM of the 50% inhibitory concentration IC₅₀ data (*n* = 10). *P*-values indicate that means were significantly different between the aspirin-sensitive asthmatics (ASA+), aspirin-tolerant asthmatics (ASA-) and normal subjects.

AA. This phenomenon was also seen in platelets from ASA+ patients but not ASA- patients (data not shown).

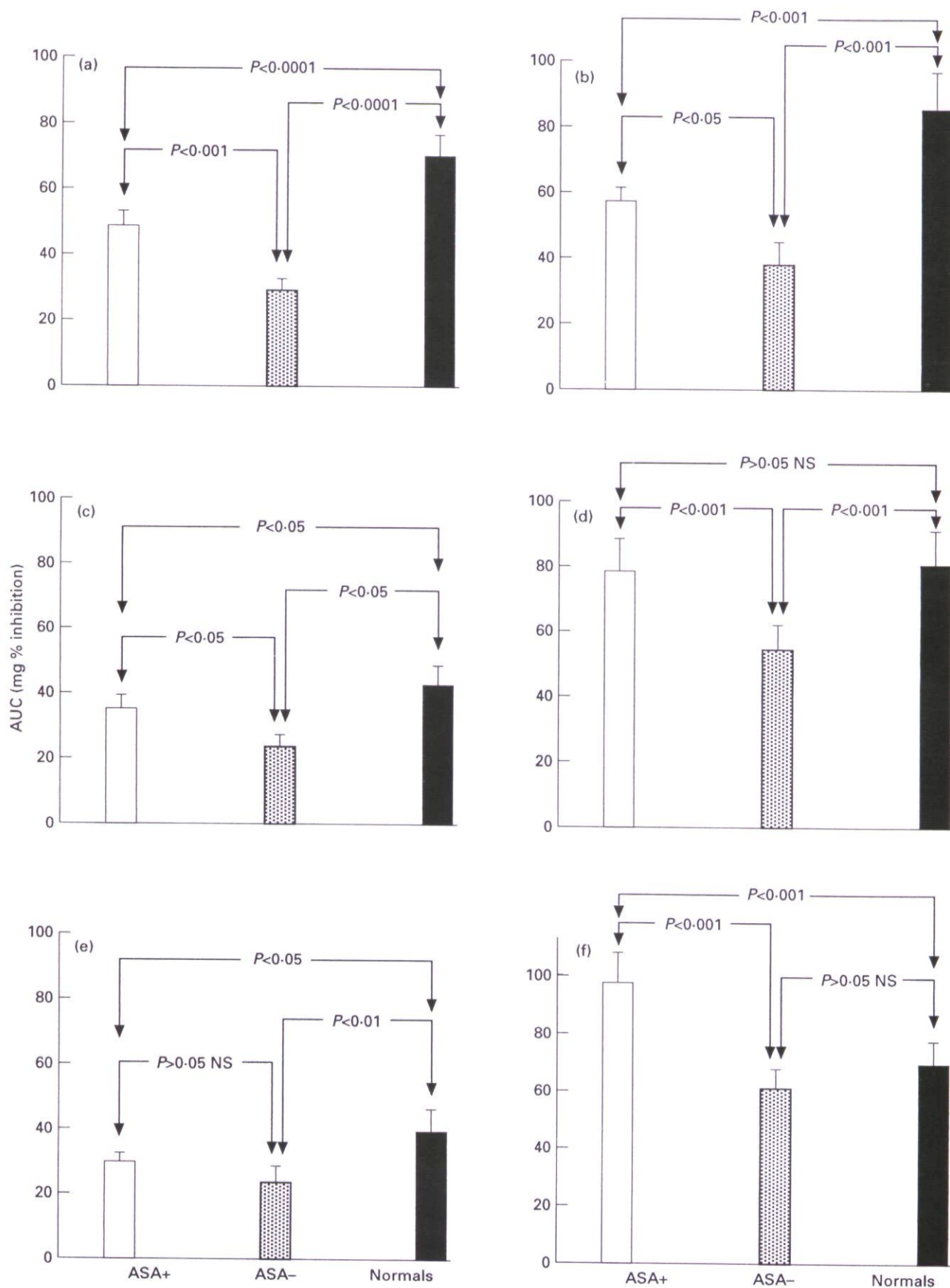
Discussion

This study has demonstrated clear differences in CD62P and CD63 expression on platelets from ASA+, and ASA- patients and normal control subjects in response to stimulation by PAF, AA and collagen suggesting differential activation in different subgroups of asthma. Thus in ASA+ patients CD62P expression induced by AA and COL was greater while CD63 expression induced by PAF and AA was decreased compared with control subjects. In ASA- patients, however, both CD62P and CD63 expression induced by PAF, AA and COL were decreased compared with control subjects and ASA+ patients. The significantly greater CD62P expression induced by AA and COL in ASA+ platelets may reflect *in vivo* priming or a dysfunction in the regulation of CD62P expression. Platelet-neutrophil

interactions are known to result in the transcellular synthesis of bronchoconstrictive mediators such as leukotriene C₄ and PAF [33]. Thus, enhanced CD62P expression on platelets is likely to facilitate CD62P mediated binding of platelets to neutrophils resulting in an influx of platelets into acute inflammatory sites and release of platelet-derived mediators thereby initiating or extending the inflammatory response [33].

In contrast to the clear differentiation between the two asthmatic groups with respect to CD62P expression, stimulation of platelets from both groups with either PAF or AA resulted in less CD63 expression compared with platelets from normal individuals. The explanation for this is unclear but if CD63 has a role in protecting plasma membranes from degradation by lysosomal hydrolases as suggested by its high density and extensive glycosylation [27], this protective effect may be reduced in asthma. It is also possible that *in vivo*, the expression of the CD63 antigen on platelets of asthmatic patients is part of the pathological process which, in turn, results in

Fig. 4. Histograms representing the inhibition by aspirin of CD63 expression induced by (a) and (b) platelet activating factor (PAF, 10 and 80 nM), (c) and (d) arachidonic acid (AA, 0.1 and 1 mM) and (e) and (f) collagen (COL, 80 and 800 µg/mL). The values are mean (± SEM)% inhibition of expression obtained by area under the curve (AUC) analysis of data from 10 aspirin-sensitive asthmatics (ASA+), 10 aspirin-tolerant asthmatics (ASA-) and 10 normal subjects.



tachyphylaxis to subsequent *in vitro* stimulation of CD63 expression. Equally, the putative role of CD63 in signal transduction processes between platelets and other cells [24] may suggest there is a dysfunction in such processes in asthma.

Classical platelet function assays were not performed in this study, but a previous study suggested that platelet aggregation to PAF is reduced in ASA+ patients compared with normal subjects while ATP release is enhanced following stimulation with PAF, AA and COL [31]. In the current study, CD62P expression induced by AA and COL was greater while CD63 expression induced by PAF was decreased in ASA+ patients. Equally, in ASA+ patients, aspirin effectively inhibited CD62P expression and ATP release but appeared to be a less effective inhibitor of CD63 expression and PAF-induced aggregation when compared with normal subjects [31]. Collectively, these results suggest that the CD62P and CD63 antigens may signal different aspects of platelet function in ASA+ and that the latter marker correlates more closely with platelet aggregation, while CD62P expression may be more closely linked with platelet secretion.

The increased platelet count and mass observed in ASA+ and ASA- patients in the present study are consistent with a previous report that platelet count, mass and bleeding time were increased in atopic compared with normal subjects [7]. These findings, together with the differences in platelet aggregation [5,7,31] and CD62P/CD63 expression reported here, strongly suggest an abnormality of platelet function associated with asthma *per se*. Significantly, and in contrast to AA and COL, CD62P expression induced by PAF was not increased in ASA+ patients and it was significantly reduced in ASA- patients when compared with normal subjects. This lack of response to *in vitro* stimulation by PAF suggests the possibility of tachyphylaxis following exposure of platelets to PAF *in vivo*, and therefore supports previous data indicating that this agonist plays an important role in the pathogenesis of asthma [34–37]. Furthermore, CD62P and CD63 expression on platelets of ASA- patients were the least inhibited by aspirin when compared with platelets from normal subjects and ASA+ patients. As such it seems likely that both CD62P and CD63 expression on ASA- platelets are already down-regulated prior to aspirin exposure and consequently less susceptible to further inhibition, particularly with PAF and AA as agonists. This provides further support for the involvement of these agonists in the pathogenesis of asthma *per se*.

The absence of any effect of aspirin on CD62P and CD63 expression in unstimulated platelet preparations is

consistent with other studies [38,39]. However, stimulation of platelets with PAF, AA or COL following incubation with aspirin demonstrated that CD62P expression on platelets from ASA+ patients was more sensitive to inhibition by aspirin than those from ASA- or normal subjects and this was most evident with maximal concentrations of the agonists. These results contrast with studies in which aspirin did not inhibit ADP and thrombin induced CD62P or CD63 expression on platelets of normal subjects [38,39]. However, the use of different platelet agonists and a lower concentration of aspirin may partly explain these apparently contradictory findings and the data on the effects of aspirin inhibition reported here are consistent with our previous observation that the effects of aspirin on platelet activation are dependent on the nature of the agonist and the dose of aspirin used [32].

The increased expression of CD62P on ASA+ platelets and subsequent increased susceptibility to inhibition by aspirin may reflect altered arachidonic acid metabolism or increased sensitivity to cyclo-oxygenase (COX) inhibition in ASA+ platelets [40]. In this regard, differential expression of the constitutive (COX-1) and inducible (COX-2) enzymes in ASA+ and the production of 15-hydroxyeicosatetraenoic acid by COX-2 in the presence of aspirin may be relevant [41,42]. Diversion of arachidonic acid from the COX to the lipoxygenase pathway and increased leukotriene generation is supported by the augmented urinary leukotriene E₄ concentrations detected in ASA+ patients both before and after aspirin challenge [43,44].

The phenomenon of two platelet populations in ASA+ following stimulation with an optimal concentration of AA confirmed previous observations on platelets from normal subjects [28]. The phenomenon was both agonist and concentration-dependent as it was not observed with PMA, PAF or COL or with submaximal concentrations of AA. These subpopulations were not observed with ASA- platelets although a kinetic study in stable atopic asthmatic patients suggested the presence of two platelet populations, one of which had a shortened lifespan [6]. Thus, one of the subpopulations observed in the present study may represent platelets undergoing 'apoptosis' or cell death with resultant swelling, fragmentation, clumping and disruption of cellular organelles [45] as, at a supramaximal concentration of AA, one of the subpopulations disappeared completely.

The results described in this study highlight that in different forms of asthma there may be differential expression and inhibition of specific platelet activation antigens that appears to be dependent on both the nature and concentration of the agonist. This suggests that fine

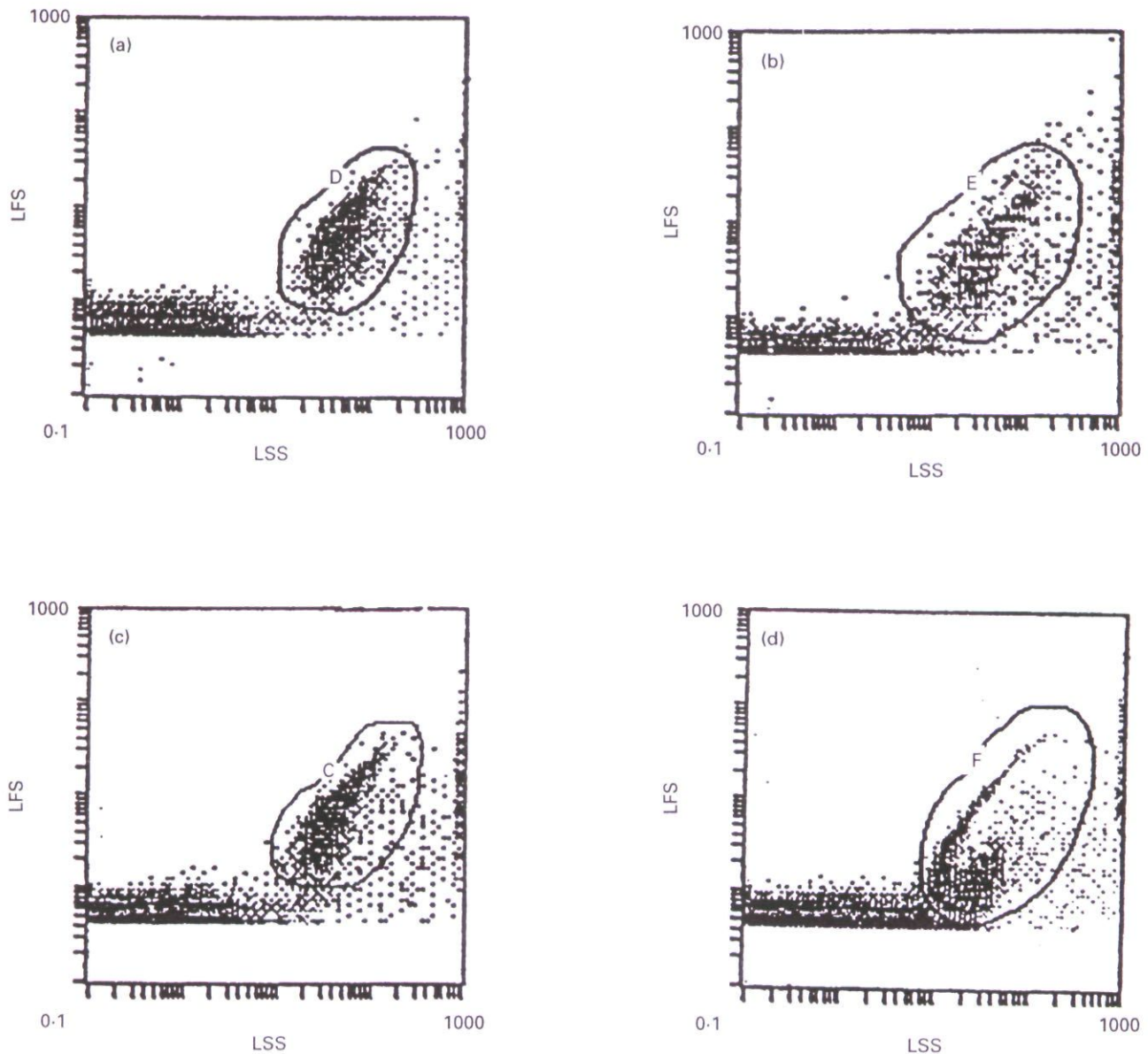


Fig. 5. Representative flow cytometric scatter profiles showing the effect of (a) platelet activating factor (PAF, 80 nM), (b) collagen (COL, 80 μ g/mL), (c) arachidonic acid (AA) (0.1 mM) and (d) AA (1 mM) in one normal subject. Two platelet populations are apparent in (d).

control of both the induction and inhibition of these adhesion receptors exists *in vivo*. The reduced expression of CD62P in ASA⁻ patients raises the possibility that this adhesion receptor exhibits tachyphylaxis possibly due to chronic stimulation *in vivo* by inflammatory mediators such as PAF. The results also suggest that platelet responses in ASA⁺ patients differ from those of ASA⁻ patients but the relevance of these differences to the pathogenesis of aspirin-sensitive asthma requires further investigation.

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