ORIGINAL ARTICLE

Nonsteroidal Anti-inflammatory Drug Hypersensitivity Syndrome: A Multicenter Study
II. Basophil Activation by Nonsteroidal Anti-inflammatory Drugs and Its Impact on Pathogenesis

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Abstract

Background: Patients who are clinically hypersensitive to nonsteroidal anti-inflammatory drugs (NSAIDs) sometimes present basophil activation in vitro, and in 50% of cases a parallel response to release of sulfidoleukotrienes (cellular allergen stimulation test) is observed. These phenomena occur not only in clinically hypersensitive patients, but also in some healthy controls who tolerate NSAIDs.
Material and Methods: We studied 16 clinically hypersensitive patients, 22 controls tolerating NSAIDs, and 29 healthy blood donors (clinical NSAID status unknown) using 2 different basophil isolation techniques (buffy coat or plasma leukocytes).
Results: In a population of 13 aspirin-tolerant healthy controls and 29 healthy blood donors, basophil activation with aspirin, diclofenac, and naproxen was analyzed at 4 different concentrations. The results in the 2 groups were quite similar in qualitative terms. Choosing a cutoff of 5% and a stimulation index >2, the proportion of positive results increased with the concentration. There were more positive results at all concentrations using the plasma leukocyte technique.
Conclusions: The most important finding of this study is that basophil activation by NSAIDs occurs not only in clinically hypersensitive patients but also, to a very variable extent and on an individual basis, in apparently normal healthy individuals who tolerate NSAIDs. The phenomenon is clearly dose-related, and hypersensitive patients seem to react to lower NSAID concentrations.

Key words: NSAID hypersensitivity. Basophil activation test. Multicenter study.

Resumen

Antecedentes: Algunos pacientes con hipersensibilidad clínica a analgésicos antiinflamatorios no esteroideos (AINES) presentan activación de basófilos tras estimulación in vitro y en un 50% de los casos se observa una respuesta paralela a la liberación de sulfidoleucotrienos (Ensayo CAST).
Este fenómeno no sólo ocurre en pacientes hipersensibles a AINES, sino que también ocurre en algunos controles sanos que toleran la administración de AINES.
Material y Métodos: Estudiamos 16 pacientes con hipersensibilidad a AINEs, 22 controles que toleran AINES y 29 sujetos sanos (con estatus clínico desconocido para AINES) y aplicamos en paralelo dos técnicas de aislamiento de basófilos diferentes (células de la interfase o buffy coat y leucocitos del plasma) en el test de activación de basófilos.
Resultados: En 13 controles sanos tolerantes a aspirina y en 22 controles sanos se analizó el TAB frente a aspirina, diclofenaco y naproxeno a cuatro diferentes concentraciones mediante ambas técnicas de separación celular, siendo los resultados cualitativamente similares en ambos grupos. Para un punto de corte de 5% e índice de estimulación >2, la proporción de casos positivos es proporcional a la concentración, siendo mayor para los leucocitos de plasma.
Conclusiones: El hallazgo más importante de este estudio fue que la activación de basófilos por AINEs es un fenómeno que ocurre no sólo en pacientes con hipersensibilidad a AINES, sino también, de forma individual en sujetos aparentemente sanos que toleran la administración de AINES. Este fenómeno está relacionado con la dosis y los pacientes hipersensibles parecen reaccionar a concentraciones más bajas de AINES.

Palabras clave: Hypersensibilidad a AINES. Test de activación de basófilos. Estudio multicéntrico.

Introduction

In recent years, several authors have reported in vitro activation of basophils by nonsteroidal anti-inflammatory drugs (NSAIDs) detected using flow cytometry [1-3], microscopy [4-6], and observation of released mediators such as sulfidoleukotrienes [6-10] or 15-HETE [11,12]. Some authors [1-3,6-8] show that this phenomenon occurs more frequently in blood cells from patients with the NSAID hypersensitivity syndrome and could therefore be used for diagnostic purposes.

In a previous article [13], we presented the results of a large multicenter study comprising 152 such patients within the framework of the European Network for Drug Allergy (ENDA). A number of observations emerged from that study. First, patients who were clinically hypersensitive to NSAIDs frequently also presented basophil activation to NSAIDs in vitro (about 60%-75% of cases), and for 50% of the cases this activation was also observed in flow-cytometric determination of the CD63 activation marker and release of sulfidoleukotrienes (cellular allergen stimulation test [CAST]). Second, these phenomena were seen to occur not only in clinically hypersensitive patients, but also in apparently healthy controls who tolerated NSAIDs. Basophil activation in healthy controls varied widely (5%-100%), and the only difference between these groups was the technique used for blood cell isolation (buffy coat or plasma leukocytes). Third, both in controls and in clinically hypersensitive patients, basophil activation by NSAIDs appears to be strictly dose-dependent.

A shift in the dose-response curve becomes apparent when hypersensitive patients and controls are taken as groups. When patients and controls are considered individually, the correlation between NSAID sensitivity in vivo and in vitro does not appear absolute, but should be further investigated. These findings have been published elsewhere [3,14-16].

In the present study, we analyze basophil activation in vitro in apparently healthy individuals who tolerate NSAIDs. We pay particular attention to the specificity of these reactions and the methodology used, namely, the cell isolation technique, in order to provide more precise information on the pathogenesis and mechanisms of NSAID hypersensitivity.

Material and Methods

Patients

The original multicenter study [13] comprised 152 patients with a history of hypersensitivity to NSAIDs recruited in 11 different groups between spring 2003 and spring 2006, and 136 controls who tolerated NSAIDs. In the present study, we analyzed the results of 16 clinically hypersensitive patients, 22 controls who tolerate NSAIDs, and 29 healthy blood donors (clinical NSAID hypersensitivity status unknown) in whom in vitro basophil activation tests (BAT) were performed in parallel using 2 different cell isolation techniques (buffy coat or plasma leukocytes).

Flow-Cytometric BAT, (Flow CAST)

All reagents (Flow CAST) and NSAID allergens were provided by the manufacturer (Bühlmann Laboratories, Allschwil, Switzerland). The technique was performed following the manufacturer's instructions and has been fully described and discussed elsewhere [13,16]. Briefly, blood was collected in EDTA tubes and stored at 4°C; the test was then carried out within 24 hours of blood sampling. One milliliter of EDTA blood allows us to test up to 2 allergens. In the plasma leukocyte isolation procedure, the tubes were first centrifuged at 200*g* for 5 minutes at 4°C. The supernatant was pipetted and recentrifuged at 500*g* for 10 minutes at 4°C. After the

supernatant was decanted, the cell pellet was resuspended in 100 µL HEPES calcium buffer (stimulation buffer) [12] per milliliter of blood. The buffy coat leukocyte isolation technique differs in the sense that whole EDTA blood is first centrifuged at 500*g* for 10 minutes, yielding a buffy coat layer that is then pipetted, washed, and centrifuged, and finally reconstituted in the same interleukin (IL) 3–containing stimulation buffer as described above (Figure 1).

Subsequently, 50 µL of reconstituted solutions of acetylsalicylic (ASA) (final concentrations 5, 2, 0.4, and 0.05 mg/mL), diclofenac (DIC) (final concentrations 1.25, 0.3, 0.06, and 0.01 mg/mL), or naproxen (NAP) (final concentration 5, 1, 0.2, and 0.05 mg/mL) was added to 50 µL of cell suspension in microplate wells. In the ENDA multicenter study, only the 2 middle concentrations were used. A monoclonal anti-immunoglobulin (Ig) E receptor antibody (Bühlmann Laboratories) at 1 µg/mL was used as a positive control.

In order to evaluate basal values without stimulation, 50 µL of stimulation buffer was added to another well and 50 µL of cell suspension added to all wells. The microplate was covered with an adhesive plastic sheet and incubated for 40 minutes at 37°C. Some groups (LIM) used tubes instead of microplates. The reaction was halted by adding a stopping buffer composed of 100 µL of HEPES buffer (pH 7.3) and EDTA (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, EDTA 0.27 mM). Soon afterwards, plates were centrifuged at 500*g* for 5 minutes at 4°C. The basophils from the cell pellet were then double labeled by adding 20 µL of staining reagent containing prediluted anti-CD63 phycoerythrin-labeled antibody and anti-IgE fluorescein-isothiocyanate (FITC)–labeled antibody. After incubation for 30 minutes at 4°C (protected from exposure to light), 4 mL of lysing reagent (BD Pharmalyse, BD Biosciences, California, USA) was added to each tube and left at room temperature for 5 minutes. Cell lysis was stopped with 1 mL of washing buffer. After centrifuging for a further 5 minutes at 1000*g*, the supernatants were decanted and 500 µL of stopping buffer (or the sheath buffer used for the cytometer) was added to the tube, which was then gently shaken before flow-cytometric analysis.

Flow-cytometric analysis was performed at 488 nm on a FACScan flow cytometer (BD Biosciences) or similar instrument equipped with 1 or 2 argon lasers. The results were analyzed using a CellQuest software program (BD Biosciences) or equivalent. On the histogram showing forward scatter and side scatter, a first cell gate was defined by a bit map around lymphocytes. A second gate was defined around cells showing high-density fluorescence with anti-IgE FITC, which identified them as basophils. In each assay, at least 500 basophils were counted. The other parameter analyzed on the identified basophils was CD63, as described elsewhere [16].

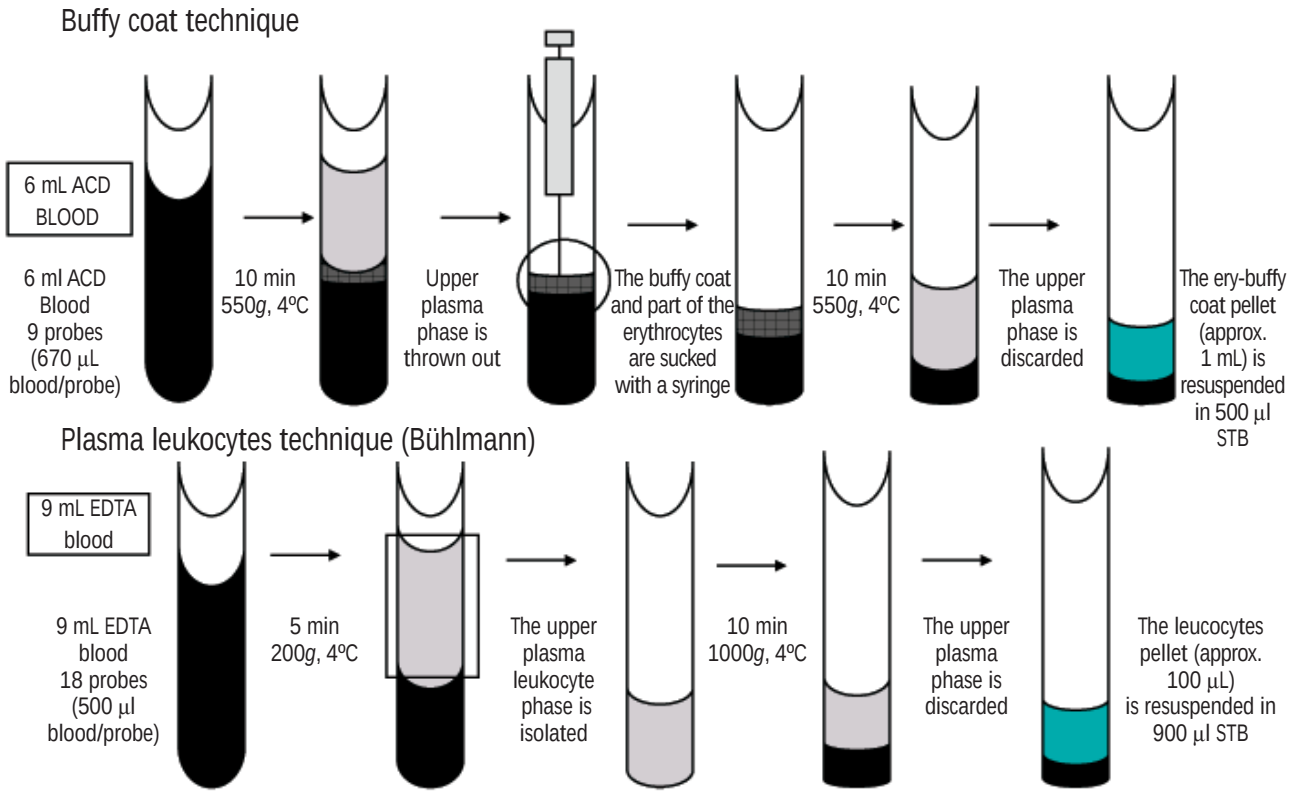

Figure 1. Schematic representation of the plasma leukocyte and buffy coat (BC) cell isolation techniques for performance of the basophil activation test.

Table 1. Number of Positive Cases in Healthy Controls in 2 Different Laboratories (Basel and Pamplona) According to the Cell Isolation Method and at 4 Different Concentrations

No. of Positive Cases From 42 Tests (BL and PAM)			Tests[b] in Basel (29 controls)			Tests[b] in Pamplona (9 controls, 13 tests)		
	Leuko	Buffy		Leuko	Buffy		Leuko	Buffy
ASA c1	26	18	ASA c1	23/29	18/29	ASA c1	4/13	2/13
ASA c2[a]	17	4	ASA c2[a]	14/29	4/29	ASA c2[a]	3/13	0/13
ASA c3[a]	10	3	ASA c3[a]	10/29	3/29	ASA c3[a]	0/13	0/13
ASA c4	8	2	ASA c4	8/29	2/29	ASA c4	0/13	0/13
DIC c1	7/13	10/13	DIC c1	ND	ND	DIC c1	7/13	10/13
DIC c2[a]	30	15	DIC c2[a]	25/29	13/29	DIC c2[a]	6/13	2/13
DIC c3[a]	17	6	DIC c3[a]	16/29	3/29	DIC c3[a]	1/13	2/13
DIC c4	10	3	DIC c4	8/29	3/29	DIC c4	2/13	0/13
NAP c1	17	30	NAP c1	14/29	19/29	NAP c1	7/13	10/13
NAP c2[a]	24	18	NAP c2[a]	24/29	10/29	NAP c2[a]	2/13	1/13
NAP c3	11	2	NAP c3[a]	11/29	2/29	NAP c3[a]	0/13	0/13
NAP c4	5	2	NAP c4	5/29	2/29	NAP c4	0/12	0/13
Total	182/475 38.3%	113/475 23.8%						

Abbreviations: ASA, acetylsalicylic acid; Buffy, buffy coat cell isolation technique; c, concentration; DIC, diclofenac; Leuko, plasma leukocyte cell isolation technique; NAP, naproxen; ND, not done.
[a]concentrations used in the ENDA study.
[b]Positivity criteria: >5% activation; stimulation index>2.

Results

We analyzed the BAT results for ASA, DIC, and NAP in a population comprising 13 ASA-tolerant healthy controls and 29 healthy blood donors (Table 1). In qualitative terms, the results for both groups were quite similar. At a cutoff of 5% and stimulation index >2, the proportion of positive results increased with the concentration and was higher at all concentrations in the plasma leukocyte population than in the buffy coat population. This explains why a difference was observed between control groups in the multicenter ENDA study and why specificity was lower in most groups using the plasma leukocyte cell isolation technique. In quantitative terms (percentage of activated basophils), the plasma leukocyte technique showed a higher dose-dependent sensitivity (Table 2) except for NAP at the highest concentration (Figure 2). Curiously, for unstimulated controls and particularly for positivity to anti-IgER antibody, basophil activation was consistently higher in buffy coat leukocytes (Figure 3).

In one group (WRO), 16 patients with a positive provocation result to ASA and 22 controls with a negative provocation result were systematically investigated in parallel (Table 3). Similar results were obtained for both groups. The mean basophil activation percentage values were markedly higher with plasma leukocytes than with buffy coat leukocytes for all 5 NSAIDs at the 2 concentrations tested. Higher activation can be observed in the healthy controls studied using plasma leukocytes than in

Table 2. Mean Basophil Activation (%) in 42 Healthy Controls

	Leuko	Buffy
Negative control	3	1
Anti-IgE	61	66
	Leuko	Buffy
ASA c1	15[a]	10
ASA c2	7	3
ASA c3	4	2
ASA c4	2	1
DIC c1	22	5
DIC c2	10	1
DIC c3	5	1
NAP c1	11	23
NAP c2	21	5
NAP c3	5	2
NAP c4	2	1

Abbreviations: ASA, acetylsalicylic acid; Buffy, buffy coat cell isolation technique; c, concentration; DIC, diclofenac; Ig, immunoglobulin; Leuko, plasma leukocyte cell isolation technique; NAP, naproxen.
[a]Mean of all tests in basophil activation (%).

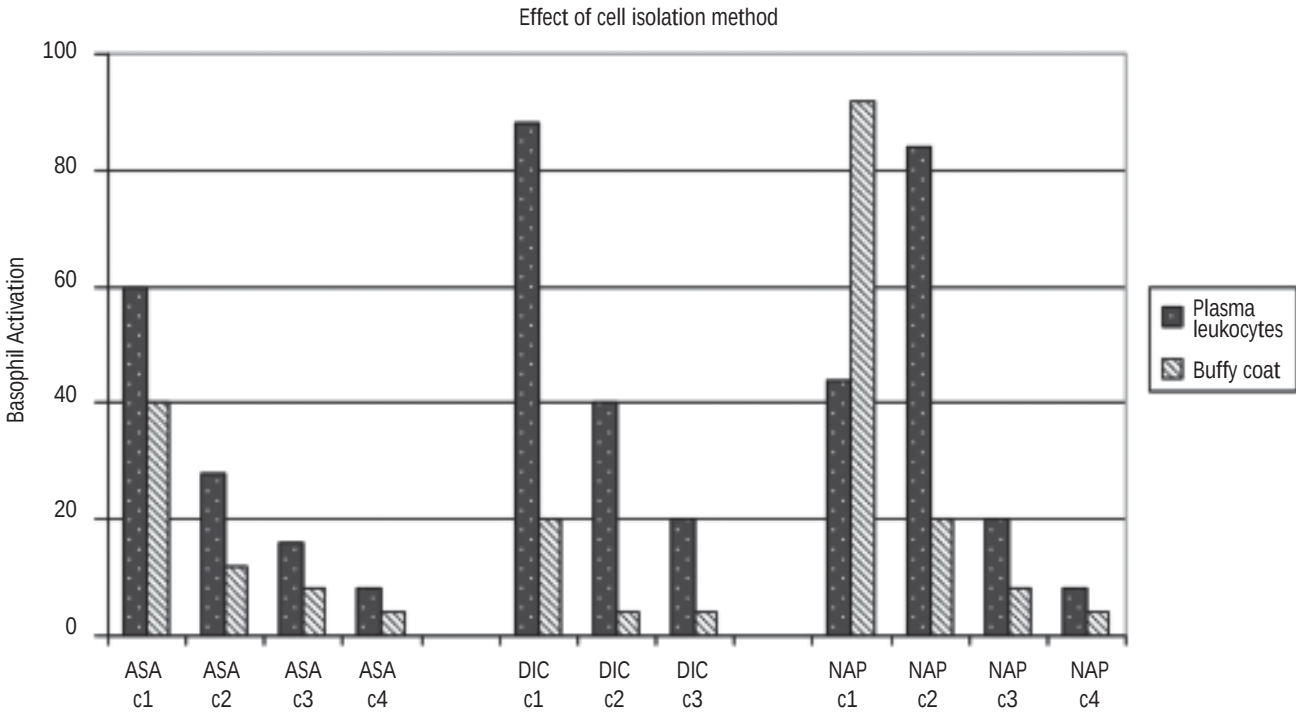

Figure 2. Effect of cell isolation technique (plasma leukocytes or buffy coat cells) on the basophil activation test (%). ASA indicates acetylsalicylic acid; c, concentration; DIC, diclofenac; NAP, naproxen.

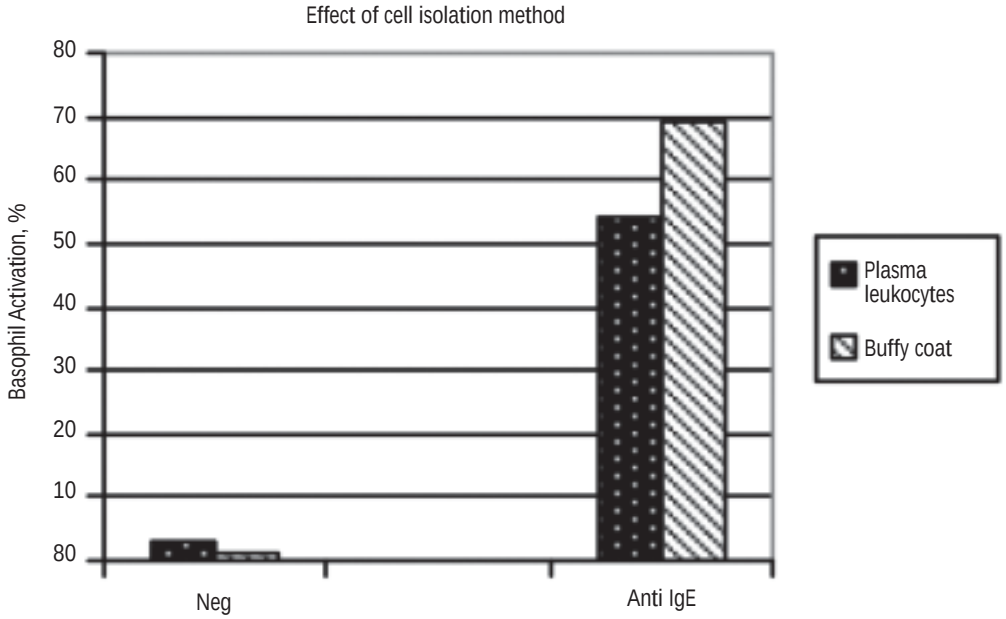

Figure 3. Effect of cell isolation technique (plasma leukocytes or buffy coat cells) on the basophil activation test (%) in negative controls and positive controls (anti-IgER1). Ig indicates immunoglobulin.

Table 3. Effect of Cell Isolation Method on Different Nonsteroidal Anti-inflammatory Drugs at 2 Concentrations in Clinically Hypersensitive Patients and in Healthy Controls

			Negative Control	Positive Control	ASA 1	ASA 0.2	PAR 1	PAR 0.2	DIC 0.3	DIC 0.06	NAP 1	NAP 0.2	MET 5	MET 0.5
Patients														
16	Plasma leukocytes	Positive[a]	1	14	12	6	4	4	14	5	13	4	3	5
		Mean percentage activity	4.81	38.44	16.44	12.12	8.06	7.81	30.44	11.36	30.17	10.40	6.41	9.81
16	Buffy coat leukocytes	Positive[a]	3	14	3	2	1	5	7	4	7	3	1	2
		Mean percentage activity	4.10	46.38	4.67	3.70	4.48	7.17	9.37	5.81	10.67	4.11	4.33	5.78
Controls														
22	Plasma leukocytes	Positive[a]	1	16	12	2	4	3	16	5	16	3	2	4
		Mean percentage activity	3.90	49.58	16.52	11.36	5.82	7.28	33.63	14.53	36.48	10.87	4.40	9.02
22	Buffy coat leukocytes	Positive[a]	4	22	7	1	0	0	9	11	7	1	3	2
		Mean percentage activity	4.18	67.40	9.91	3.64	3.16	4.26	11.87	8.84	15.49	4.19	5.21	8.02

Abbreviations: ASA, acetylsalicylic acid; c, concentration; DIC, diclofenac; MET, metamizole; NAP, naproxen; PAR, paracetamol.
[a] Positivity criteria: >5% activation; stimulation index >2.

the healthy controls studied using the buffy coat [13] (Table 4). A detailed case-by-case analysis (results not shown) confirms what is described below: ASA-positive patients and controls are mostly also positive to DIC and NAP. Paracetamol and metamizole yield only low numbers of positive cases.

Listing individual results for 42 controls with ASA (Table 4), DIC (Table 5), and NAP (Table 6), it became evident that dose-dependent basophil sensitivity is an individual characteristic of each patient tested, and may be reproduced with plasma leukocytes or buffy coat leukocytes, the latter being less sensitive. This is also confirmed in quantitative terms when analyzing quantitative correlations between reactions to ASA, DIC, or NAP in plasma leukocyte or buffy coat leukocyte cell populations (Figure 4). The correlation coefficients observed are rather high (ASA, r=0.58; DIC, r=0.67; NAP, r=0.70).

A comparison of the reactions to ASA, DIC, and NAP in all participants (Table 7) also revealed that individual dose-dependent reactivity manifests itself in parallel to the 3 NSAIDs tested. This is also clear in quantitative terms, since high reactivity to one NSAID is correlated with high reactivity to another (Figure 5) (eg, ASA/DIC, r=0.67; ASA/NAP, r=0.79; DIC/NAP, r=0.69).

In contrast, there appears to be no correlation whatsoever between basophil reactivity to NSAIDs and spontaneous basophil reactivity or reactivity induced by anti-IgER antibody.

These values correlate well when evaluated with plasma leukocytes and buffy coat leukocytes (Figure 6), thus establishing confidence in the results. However, there seems to be no correlation whatsoever (using plasma leukocytes) between basophil reactivity to ASA, DIC, or NAP and reactivity to anti-IgER antibody (positive control) (ASA, r=0.14; DIC, r=0.25; NAP, r=0.06) (Figure 7).

Discussion

Spontaneous reactivity and reactivity to IgE-mediated stimuli (so called releasability) vary from one individual to another [17-20]. Even if the mechanisms of basophil activation by NSAID-mediated and IgE-mediated stimuli are not linked, we might wonder, on the basis of theoretical considerations discussed at greater length below, whether they can influence each other. A decrease in

Table 4. Effect of Cell Isolation Method on Basophil Activation Test to Acetylsalicylic Acid at 4 Concentrations in 42 Individual Healthy Controls

		Basel (Plasma Leukocytes)			Pamplona (Buffy Coat)				
	Aspirin 5 mg/mL	1 mg/mL	0.2 mg/mL	0.05 mg/mL	5 mg/mL	1 mg/mL	0.2 mg/mL	0.05 mg/mL	Control Number
	55**	23	5	3	57	7	3	6	BL1
	112	53	37	4	33	5	6	2	2
	306	131	70	57	145	34	36	3	3
	268	84	41	40	125	27	24	7	4
	252	114	68	38	304	46	28	29	5
	176	119	97	20	149	46	28	28	6
	191	72	22	27	125	38	20	13	7
	25	17	12	6	15	7	7	7	8
	52	39	36	32	52	17	24	17	9
	86	43	13	0	13	33	5	11	10
	10	12	0	0	30	23	7	19	Pam1
	62	0	0	0	104	28	11	8	2
	0	3	0	3	37	10	11	4	3
	0	0	5	7	21	19	15	26	4
	0	73	17	29	0	7	15	11	5
	34	13	23	8	13	0	14	0	6
	5	13	3	0	4	0	4	0	7
	7	7	8	0	5	7	0	0	8
	28	0	4	8	110	39	4	6	9
	116	115	9	7	21	3	42	17	10
	98	42	21	20	45	22	5	0	11
	20	97	8	6	18	24	44	15	12
	83	24	9	14	21	0	8	0	13
	75	19	11	7	19	3	3	17	BL 11
	27	22	9	17	18	7	7	9	12
	34	37	35	15	51	16	23	22	13
	28	35	8	9	12	6	12	4	14
	60	52	26	23	88	16	11	9	15
	190	100	89	65	94	19	13	14	16
	364	130	73	89	317	82	39	28	17
	316	194	78	67	300	59	29	25	18
	305	155	54	147	303	29	13	4	19
	92	41	62	30	237	43	408	357	20
	31	45	39	70	28	147	153	23	21
	103	34	24	24	185	34	10	10	22
	203	81	64	57	133	39	23	14	23
	40	18	20	7	35	18	13	8	24
	105	35	23	5	50	13	5	10	25
	78	60	27	8	28	13	5	5	26
	50	34	23	7	83	15	0	0	27
	143	35	15	18	125	28	23	18	28
	255	251	255	238	200	178	220	213	29
Mean Pam[a]	3.6	3.1	0.8	0.8	3.3	1.4	1.4	0.8	
Mean total[b]	108	59	34	29	89	29	33	24	
Pos/42	26	17	10	8	18	4	3	2	

Abbreviations: BL, Basel; DIC, diclofenac; Pam, Pamplona
[a]Results in activation percentage.
[b]Results in activation percentage ×10

prostaglandin E_2, a natural brake of basophil activity [21-25], might eventually stimulate spontaneous and/or IgE-mediated activation. A similar effect could be due to a decrease in levels of prostanoid receptors [25] or other anti-inflammatory prostanoids such as thromboxane A_2 [26] or lipoxins [27,28]. This would explain some allergic reactions that seem to require simultaneous occurrence of an IgE-mediated stimulus and NSAID administration [29-31]. Therefore, in a pilot study, we determined whether basophil reactivity in vitro to the *Dermatophagoides pteronyssinus* in house dust mite–allergic patients is influenced by different doses of NSAIDs. As seen in Figure 8, this does not appear to be the case. The highest dose of DIC appears to reduce

Table 5. Effect of Cell Isolation Method on the Basophil Activation Test to Diclofenac at 4 Concentrations in 42 Healthy Controls

	Basel (Plasma Leukocytes)				Pamplona (Buffy Coat)				
	1.25 mg/mL	0.3 mg/mL	0.06 mg/mL	0.01 mg/mL	1.25 mg/mL	0.3 mg/mL	0.06 mg/mL	0.01 mg/mL	
		165[b]	16	17		34	27	18	BL1
		140	77	31		21	15	7	2
		441	148	80		76	30	35	3
		379	171	64		71	162	40	4
		519	180	84		138	31	27	5
		67	194	87		58	42	21	6
		235	80	49		64	8	17	7
		0	20	6		16	0	0	8
		94	46	26		28	22	10	9
		140	99	13		28	14	3	10
	156	6	5	0	603	29	15	23	Pam1
	425	14	0	0	710	26	7	7	2
	126	6	6	6	871	26	56	0	3
	0	12	7	7	722	17	92	9	4
	3	75	12	43	59	19	15	35	5
	666	51	17	16	49	27	5	5	6
	60	19	7	6	178	35	17	5	7
	333	74	35	5	88	47	6	6	8
	31	31	5	0	141	28	8	0	9
	328	65	8	71	392	307	9	13	10
	0	721	94	80	22	310	4	18	11
	0	6	7	21	0	36	7	11	12
	0	41	0	23	29	64	6	5	13
		74	21	13		12	5	11	BL 11
		63	28	8		18	6	11	12
		69	40	14		30	14	10	13
		30	25	21		0	0	5	14
		106	48	31		37	7	10	15
		127	105	77		76	33	17	16
		113	103	93		94	20	40	17
		511	276	116		185	42	32	18
		458	149	41		93	31	18	19
		133	73	23		91	442	318	20
		77	93	47		8	50	98	21
		244	55	33		107	19	4	22
		186	98	42		56	24	33	23
		38	20	7		45	17	20	24
		90	43	30		38	8	3	25
		65	43	10		48	6	10	26
		33	35	20		19	0	0	27
		95	17	20		42	13	14	28
		680	306	288		638	285	275	29
Mean Pam[a]	16.4	8.6	1.6	2.1	29.7	7.5	1.9	1.1	
Mean total[b]	154	67	40			75	39	30	
Pos/42	7/13	30	17	10	10/13	15	6	3	

Abbreviations: BL, Basel; DIC, diclofenac; Pam, Pamplona; Pos, positive.
[a]Results in activation percentage
[b]Results in activation percentage × 10

D pteronyssinus–induced basophil activation), and the lower DIC concentrations have no booster effect.

The most important finding of our study is that basophil activation by NSAIDs occurs not only in clinically hypersensitive patients, but also, to a very variable extent and on an individual basis, in healthy individuals who tolerate NSAIDs. The phenomenon is clearly dose-related: hypersensitive patients seem to react to lower NSAID concentrations. Clinical hypersensitivity to NSAIDs therefore seems to be related to a shift in the dose-response curve.

Table 6. Effect of Cell Isolation Method on Basophil Activation Test to Naproxen at 4 Concentrations in 42 Individual Healthy Controls

	Basel (Plasma Leukocytes)				Pamplona (Buffy Coat)				
	5 mg/mL	1 mg/mL	0.2 mg/mL	0.05 mg/mL	5 mg/mL	1 mg/mL	0.2 mg/mL	0.05 mg/mL	
	18[b]	135	5	8	89	24	9	5	BL1
	28	182	22	6	15	11	3	0	2
	329	320	79	37	497	73	36	32	3
	124	222	47	22	263	72	14	18	4
	276	443	110	37	532	87	31	33	5
	109	352	110	30	439	151	44	16	6
	119	153	84	20	0	0	0	0	7
	22	25	26	14	0	0	0	0	8
	97	181	26	9	0	0	0	0	9
	22	75	26	11	0	0	0	0	10
	136	6	35	27	639	36	13	0	Pam1
	183	0	4	9	528	41	0	8	2
	77	3	6	6	787	10	14	14	3
	14	8	5	0	298	20	14	25	4
	12	24	38	8	25	4	18	8	5
	32	21	12	4	131	19	9	4	6
	6	9	0	3	87	37	0	5	7
	ND	41	5	0	239	6	6	13	8
	204	94	0	0	724	48	0	4	9
	202	23	32	0	741	83	9	0	10
	318	289	16	23	285	15	15	5	11
	20	4	7	11	26	16	6	18	12
	16	21	22	4	84	64	6	16	13
	32	64	10	11	58	77	5	2	BL 11
	19	77	27	14	41	36	9	4	12
	35	36	19	17	62	49	11	11	13
	5	49	14	6	12	63	4	0	14
	54	63	19	11	135	177	16	6	15
	45	177	83	12	117	263	12	19	16
	97	263	118	66	433	467	17	28	17
	228	467	232	64	438	302	22	17	18
	99	302	100	67	237	80	13	6	19
	138	80	50	26	215	75	326	321	20
	23	75	75	54	90	211	24	17	21
	102	211	36	37	255	91	14	6	22
	97	91		26	195	22	18	15	23
	20	22	8	45	40	60	12	7	24
	32	60	22	8	50	58	10	5	25
	33	58	27	10	67	42	5	2	26
	8	42	8	13	0	72	10	26	27
	32	72	17	18	62	251	10	22	28
	255	251	255	238	200	178	220	213	29
Mean Pam[a]	10.3	4.2	1.1	0.7	35.3	2.7	2.7	0.9	
Mean total[b]	88	120	52	33		190	117	33	32
Pos/42	17	24	11	5		30	18	2	2

Abbreviations: BL, Basel; ND, not determined; Pam, Pamplona; Pos, positive.
[a] Results in activation percentage
[b] Results in activation percentage × 10

The most probable hypothesis is that both clinical hypersensitivity and basophil reactivity are related to the pharmacological effects of NSAIDs. Both phenomena seem to be qualitatively and quantitatively correlated with NSAIDs such as ASA, DIC, and NAP. However, we show that there is no correlation between basophil reactivity to NSAIDs and that induced by an IgE-dependent mechanism.

It seems reasonable to consider that a link exists between basophil activation and the symptoms of NSAID hypersensitivity: increased levels of LTC_4 have been observed

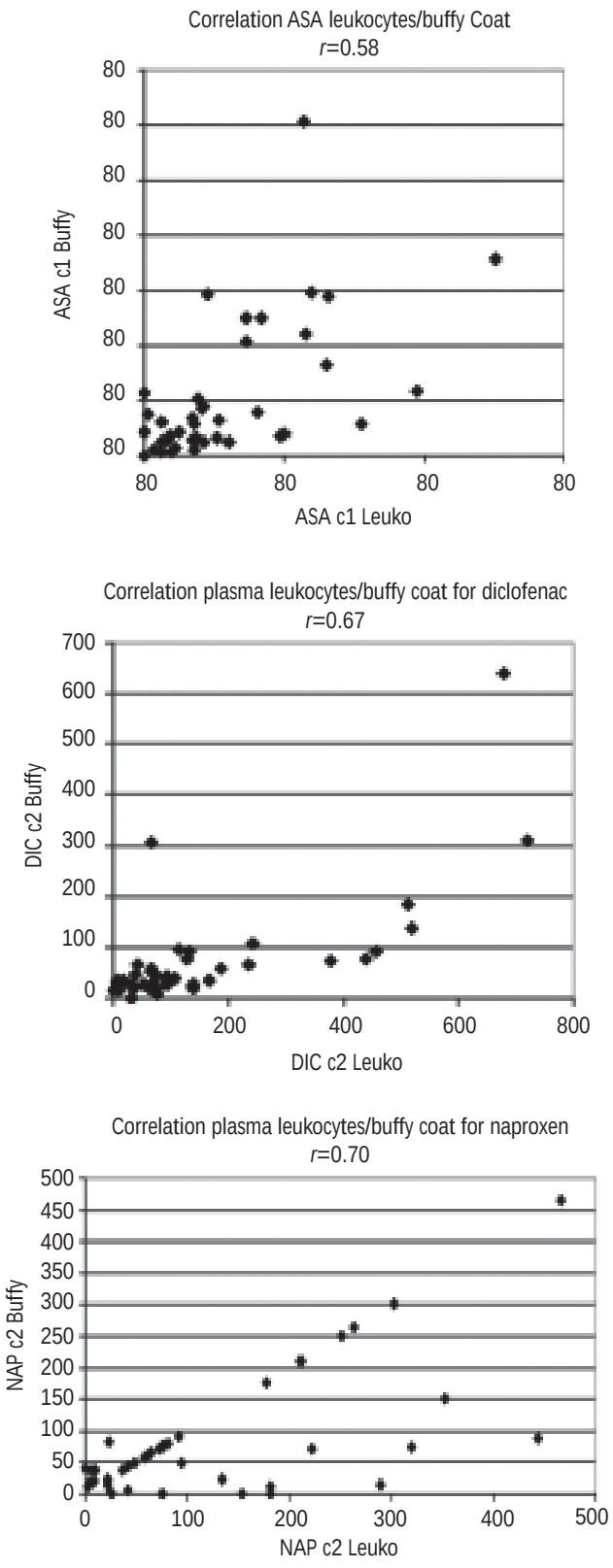

Figure 4. Correlation between BAT reactivity (% ×10) in plasma leukocytes and buffy coat cells to acetylsalicylic acid, diclofenac, and naproxen in 29 healthy controls.

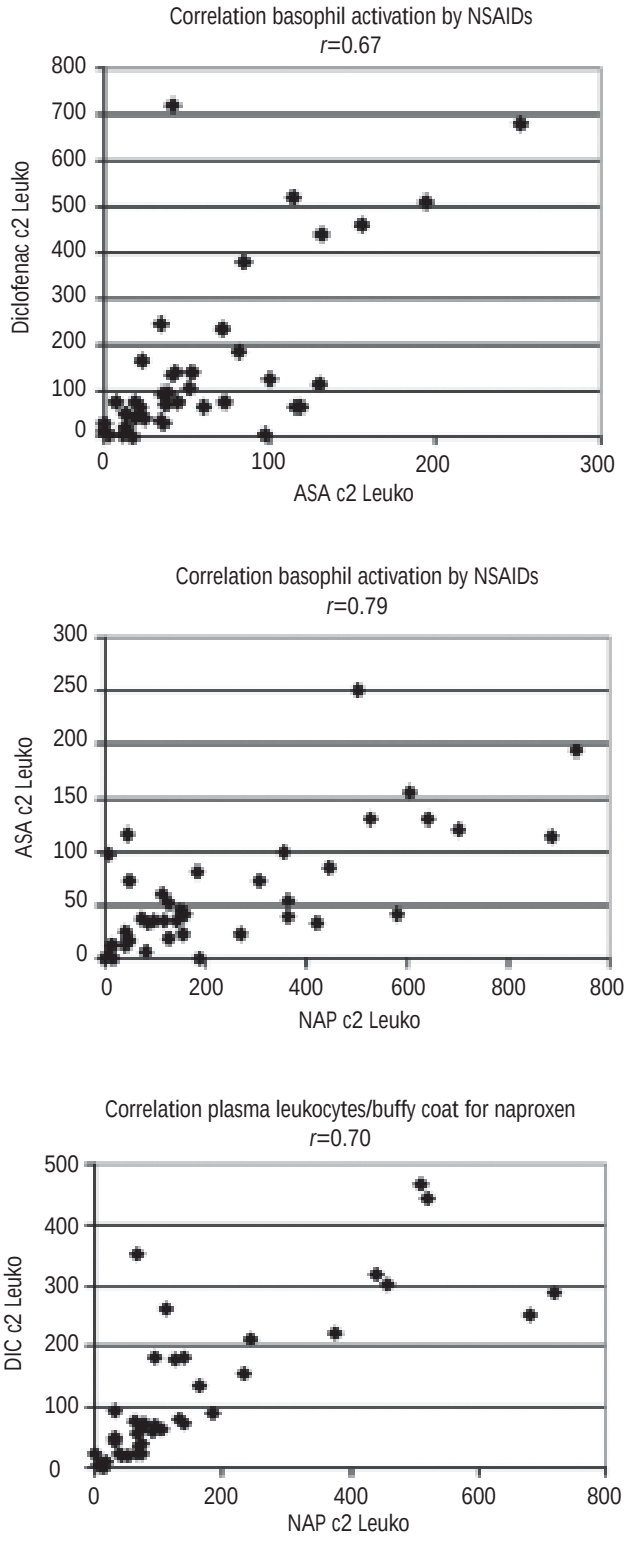

Figure 5. Correlation between acetylsalicylic acid, diclofenac, and naproxen for basophil activation test reactivity (%×10) based on plasma leukocytes.

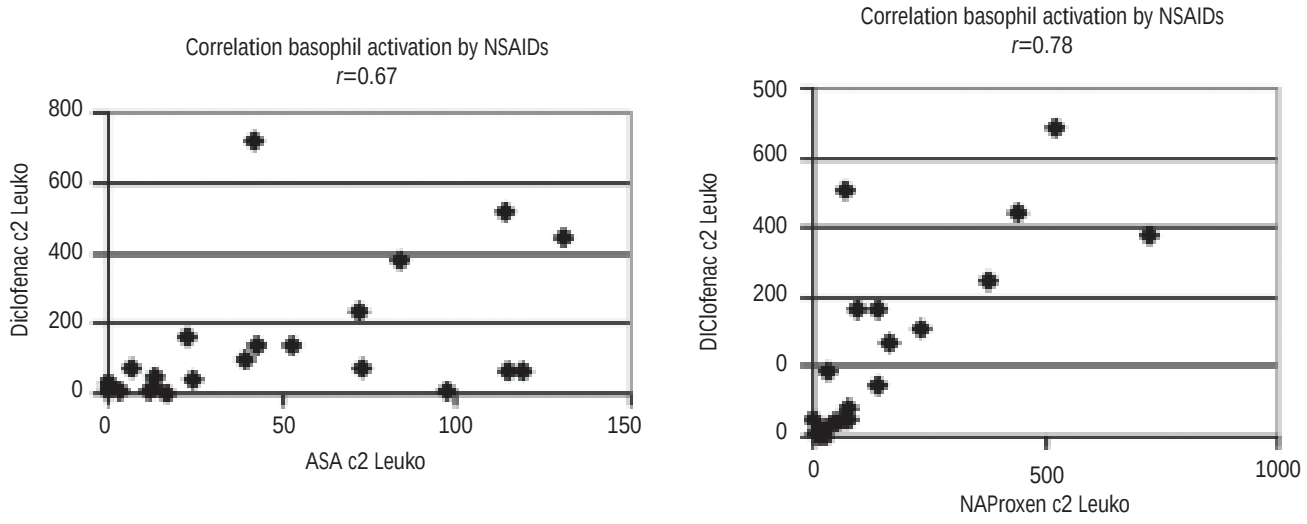

Figure 6. Correlation between plasma leukocytes and buffy coat leukocytes for negative controls and positive controls (anti-IgER) in 42 healthy individuals.

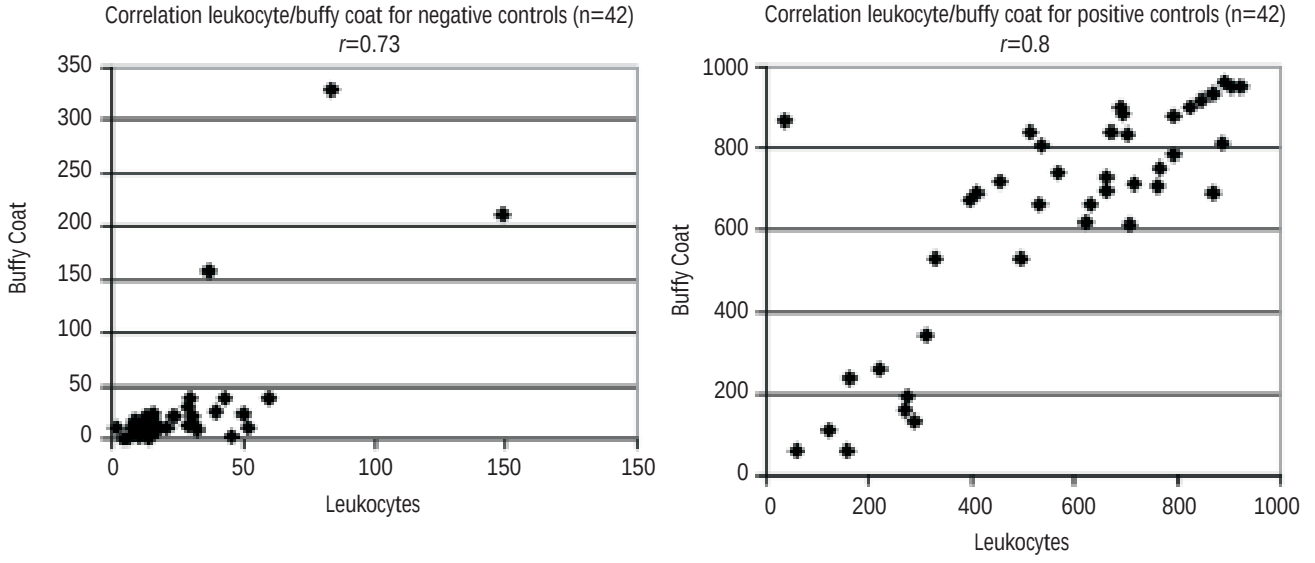

Figure 7. Correlation between acetylsalicylic acid, diclofenac, and naproxen and positive anti-IgER control in the plasma leukocytes of 42 healthy individuals.

in various body fluids and tissues [32-36]. Our study and others [37,38] show that increased release of LTCs in vitro frequently accompanies NSAID-induced basophil activation. Kowalski et al [11,12] have also reported that 15-HETE is an additional mediator in these reactions. Interestingly, in a preliminary study on their 11 hypersensitive patients and 10 controls, the authors also found a correlation between the BAT results and the 15-HETE released from blood cells (Figure 9; detailed results not shown). These preliminary findings warrant further study.

Despite the above, the precise molecular and cellular mechanisms leading to basophil activation by NSAIDs remain hypothetical. It is tempting to speculate that basophil activation in this case is essentially a consequence of inhibition of prostaglandin synthesis, particularly of prostaglandin E_2, which has been shown to be an inhibitor of NSAID-induced clinical hypersensitivity symptoms [39]. When prostaglandin E_2 synthesis is inhibited, basophil activation and the corresponding release of mediators may be enhanced [22,23]. However, the primary

Table 7. Comparative Basophil Activation Test Results for Acetylsalicylic Acid, Diclofenac, and Naproxen in Plasma Leukocytes and Buffy Coat in 42 Individual Patients

ASA c2 Leuk	ASA c1 Buffy	DIC c2 c2 Leuk	DIC c2 c2 Buffy	NAP c1 c1 Leuk	NAP c1 c1 Buffy	NAP c2 c2 Leuk	NAP c2 c2 Buffy	ASA c2 Leuk
1	57	165	34	18	89	135	24	23
53	33	140	21	28	15	182	11	53
131	145	441	76	329	497	320	73	131
84	125	379	71	124	263	222	72	84
114	304	519	138	276	532	443	87	114
119	149	67	58	109	439	352	151	119
72	125	235	64	119	0	153	0	72
17	15	0	16	22	0	25	0	17
39	52	94	28	97	0	181	0	39
43	13	140	28	22	0	75	0	43
12	30	6	29	136	639	6	36	12
0	0	14	26	183	528	0	41	0
3	37	6	26	77	787	3	10	3
0	21	12	17	14	298	8	20	0
73	104	75	19	12	25	24	4	73
13	13	51	27	32	131	21	19	13
13	4	19	35	6	87	9	37	13
7	5	74	47	124	239	41	6	7
0	21	31	28	204	724	94	48	0
115	110	65	307	202	741	23	83	115
42	45	721	310	318	285	289	15	42
97	18	6	36	20	26	4	16	97
24	21	41	64	16	84	21	14	24
19	3	74	12	32	58	64	64	19
22	7	63	18	19	41	77	77	22
37	16	69	30	35	62	36	36	37
35	6	30	0	5	12	49	49	35
52	16	106	37	54	135	63	63	52
100	19	127	76	45	117	177	177	100
130	82	113	94	97	433	263	263	130
194	59	511	185	228	438	467	467	194
155	29	458	93	99	237	302	302	155
41	43	133	91	138	215	80	80	41
45	147	77	8	23	90	75	75	45
34	34	244	107	102	255	211	211	34
81	39	186	56	97	195	91	91	81
18	18	38	45	20	40	22	22	18
35	13	90	38	32	50	60	60	35
60	13	65	48	33	67	58	58	60
34	15	33	19	8	0	42	42	34
35	28	95	42	32	62	72	72	35
251	178	680	95	255	200	251	251	251
2450	2212							
	r=0.58 ASA c1 Leuk/Buffy		r=0.69 ASA2/NAP2 Leuk		r=0.61 DICc2/Leuk/Buffy		r=0.79 DIc2/NAP2 Leuk	

Abbreviations: ASA, acetylsalicylic acid; Buffy, buffy coat cell isolation technique; c, concentration; DIC, diclofenac; Leuk, plasma leukocyte cell isolation technique; NAP, naproxen.

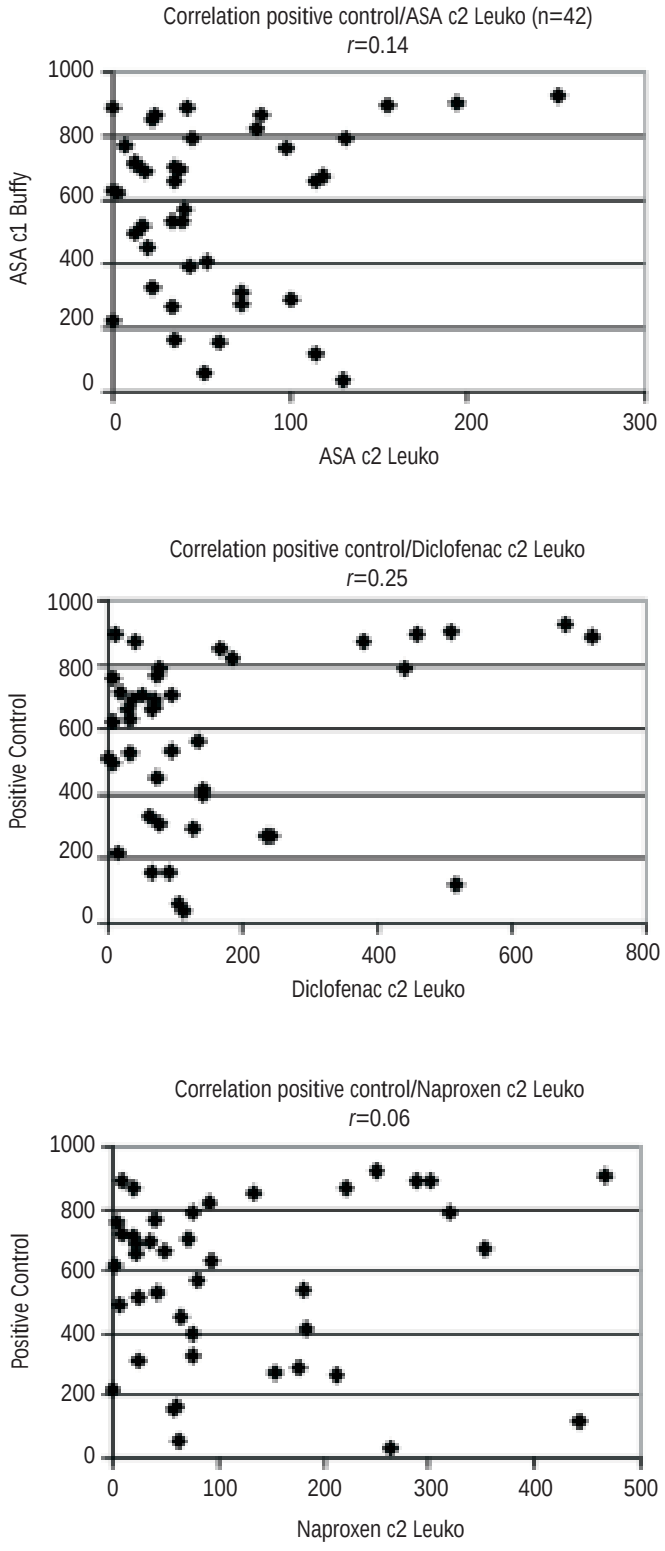

Figure 8. Effect of various concentrations of diclofenac on the basophil reactivity to the D1 allergen in 11 individuals who were allergic to house dust mites. Notice the toxic effect of diclofenac at the highest concentration.

trigger remains unknown, and it has not been established why the dose needed for this pharmacological effect differs between NSAID-intolerant and NSAID-tolerant patients, and even among NSAID-tolerant patients. Pharmacological sensitivity to NSAIDs, in terms of prostaglandin inhibition, may differ sharply, even among healthy individuals [40,41], although it has received little attention. For example, the dose-response curve for prostaglandin synthesis inhibitors shows very marked individual differences [40], which may even express themselves clinically as aspirin resistance [42] or subclinical aspirin sensitivity [43]. An inverse relationship between LTC_4 release by basophils and prostaglandin E_2 synthesis in blood cells in the presence of NSAIDs in vitro has also been observed [44,45], and a deficiency in prostaglandin E_2 synthesis and/or receptors has been repeatedly reported in aspirin-hypersensitive patients [46-49].

On the one hand, we must ask whether pharmacological sensitivity to NSAIDs is a fixed and permanent genetically determined value for a given individual [50-57]: numerous genes [53-56] have been reported to be associated with NSAID hypersensitivity and familial aggregation [57]. On the other hand, pharmacological sensitivity to NSAIDs may be a variable parameter, which changes and fluctuates with time. There are several arguments in favor of the second interpretation: a) the occurrence of NSAID hypersensitivity, mainly later in life; b) the frequently fluctuating nature of hypersensitivity, since positive provocation results are often followed by negative ones [58,59]; c) the relationship with some previous states of chronic inflammation either in the airways (rhinitis, asthma) or on the skin (urticaria), thus explaining the distinct topical locations of the clinical manifestations, despite a similar cellular and mediator mechanism.

One of the better-known effects of chronic inflammation on human basophils is increased sensitivity to C5a, which is manifested by an increased density of C5a receptors [60-62] and/or an intracellular priming effect [63,64]. Indeed, increased sensitivity to C5a has also been reported in the blood cells of patients who are hypersensitive to NSAIDs [10,65,66]. It has also been determined that quantitative sensitivity to C5a and basophil reactivity to various stimuli were correlated in various categories of patients [64]. Indeed, the study of the role of C5a as a possible trigger in NSAID-hypersensitive patients, together with inhibition of prostaglandin E_2 synthesis has been abandoned too soon. A group of experts has reported short-term complement activation and C5a generation upon administration of NSAIDs [67,68]. If this occurs in an individual who is particularly sensitive to the pharmacological prostaglandin E_2–depleting and basophil activation–increasing effect of NSAIDs, the occurrence of a reaction is readily understandable. The study [69] that failed to confirm the German report should not be considered as a definitive rebuttal, as is usually the case with reviewers [72-74], especially as recent reports confirm the increase in C5a levels after administration of NSAIDs [70,71]. It would indeed be interesting to perform a long-term longitudinal study of C5a sensitivity, dose-related basophil reactivity to

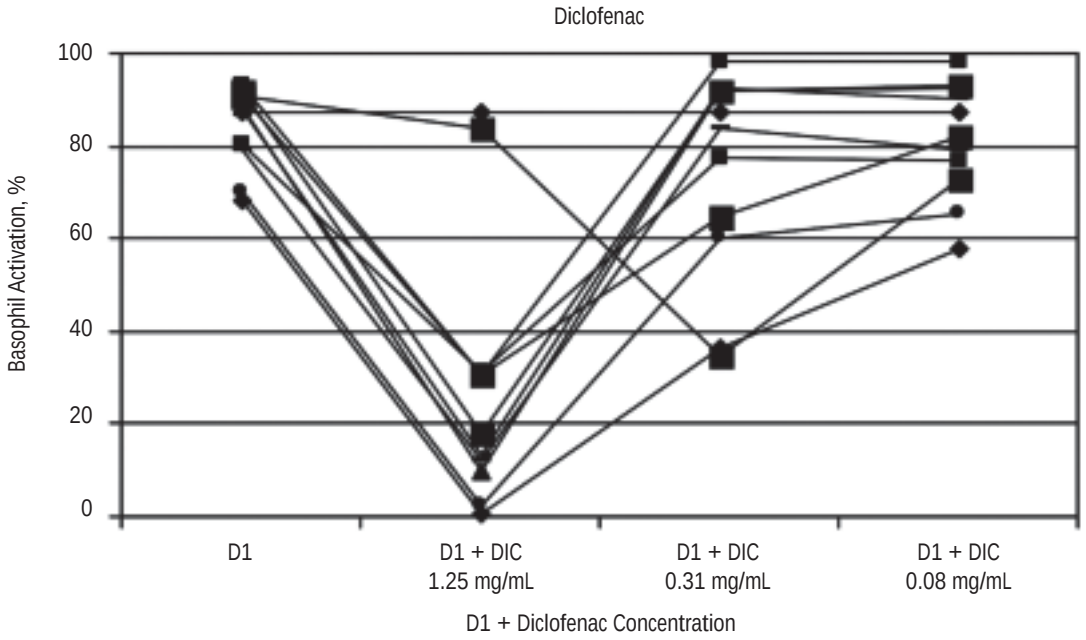

Figure 9. Effect of various concentrations of diclofenac on the basophil reactivity to the D1 allergen in 11 individuals who were allergic to house dust mites. Notice the toxic effect of diclofenac at the highest concentration.

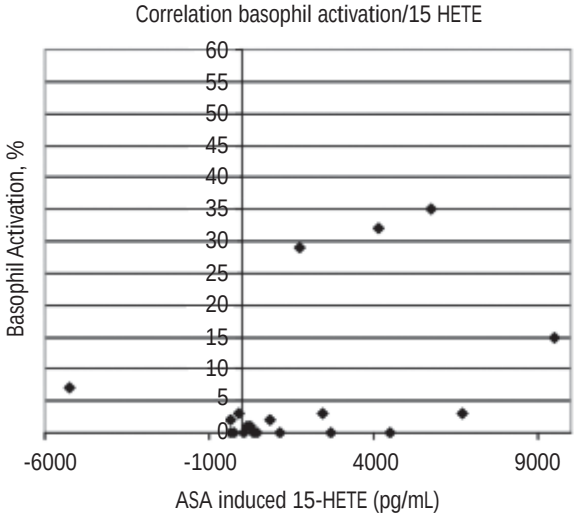

Figure 10. Correlation between basophil activation with ASA and 15-HETE synthesis in 11 patients with NSAID-hypersensitive asthma and 10 patients with NSAID-tolerant asthma (ENDA study; Lodz, unpublished results).

NSAIDs, and inhibition of prostaglandin synthesis in various categories of individuals (healthy, asthmatics, urticaria) who were clinically tolerant or intolerant to NSAIDs, in order to establish fluctuation and correlations between the clinical and biological parameters.

Reviews frequently state that the mechanism of NSAID hypersensitivity is poorly understood [72-76] or simply "unknown" [77]. We feel that this view is too pessimistic. NSAID hypersensitivity seems more likely to be the result of several concurring factors. The first factor is the occurrence of local inflammatory phenomena, which could increase the reactivity (releasability) of local mast cells and may also influence the reactivity of blood basophils (eg, C5a receptor density) and eosinophils. There is still some dispute about the role of eosinophils, which are abundant in nasal polyps [78-80] and asthmatic bronchi and an important source of LTCs, and that of mast cells. There is strong evidence that mast cells and their mediators are also directly involved in reactions to ASA [80-86]. However, pre-existing phenomena of local inflammation seem a more plausible explanation for the localization of symptoms to the airways or to the skin in NSAIDs hypersensitivity. But what are the causes of local inflammation? There is some evidence that these cause are manifold, such as viral infection [87-89], autoimmune phenomena [90-91], or bacterial infection [92-94]. Other cell types, such as epithelial cells [95] and T lymphocytes [96-99], also seem to be involved. It is difficult to distinguish between primary inflammation, which could be responsible for the development of local NSAID hypersensitivity, and secondary inflammation, which is the direct consequence of NSAID-induced cellular reactions and mediator release (Figure 10).

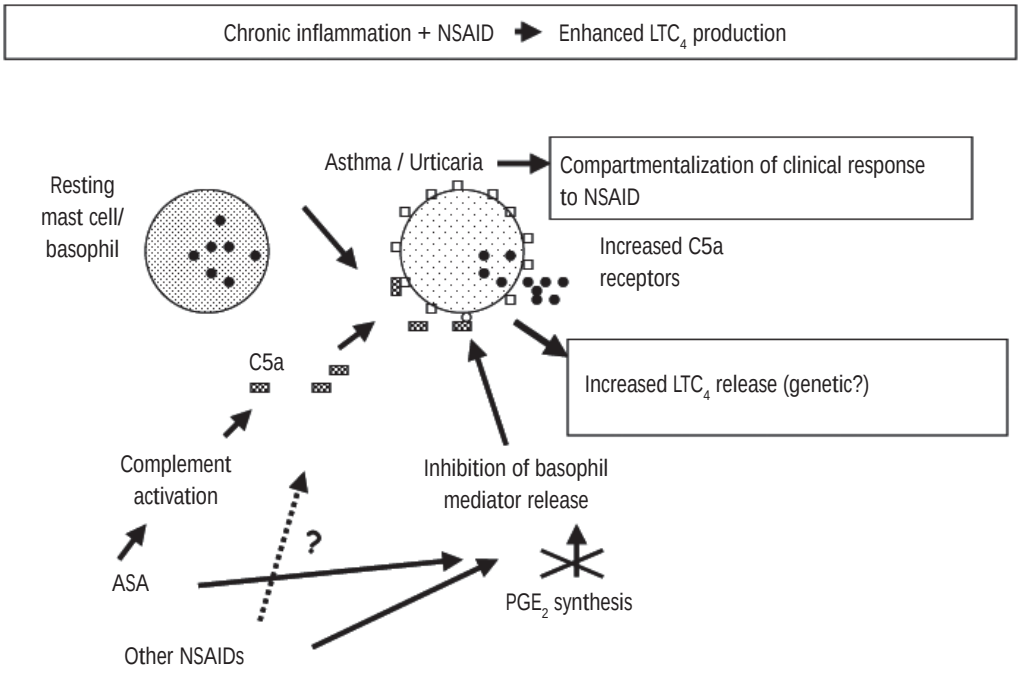

Figure 11. An integrated view of NSAID hypersensitivity. Localized chronic inflammation in the airways or on the skin enhances basophil and mast cell reactivity, resulting in an increase in C5a receptors and cell priming. NSAID-induced inhibition of prostaglandin E_2 synthesis removes a natural brake of mediator release; this effect may be accentuated by a constitutive decrease in prostaglandin E_2 receptors and prostaglandin E_2 synthesis on a genetic basis. Short-term activation of complement and generation of C5a may provide a trigger for highly reactive mast cells and basophils.

The second contributing factor is the individual pharmacological effect of NSAIDs on prostaglandin synthesis of inflammatory cells, including basophils and mast cells. Circulating basophils are probably not the major effectors of clinical symptoms in NSAID hypersensitivity, but are mostly local and probably more influenced by mast cells [80-86], and possibly by infiltrated eosinophils and basophils. However, circulating basophils may serve as indicators, and there is evidence that local reactions to NSAIDs also have systemic effects in some patients [100]. However, absolute parallelism between clinical local NSAID hypersensitivity and in vitro blood basophil reactivity should not be expected in terms of pathophysiology, although it does explain the "false-negative" reactions to the BAT. A schematic representation of these views on the pathogenesis of NSAID hypersensitivity is shown in Figure 11.

The questions yet to be answered in order to fully establish the postulated mechanism are as follows:

1. Is there a direct link between local changes in effector cells (eg, mast cells, eosinophils, epithelial cells) caused by chronic inflammation and shifts in pharmacological dose-response to NSAIDs?

2. Are basophils only markers or also effectors in clinical symptoms to NSAIDs? Is there some quantitative correlation between in vivo hypersensitivity to NSAIDs and basophil reactivity in vitro?

3. What is the molecular and intracellular cascade linking the various pharmacological effects of NSAIDs, basophil activation, and, ultimately, mediator release?

The answers to these questions require simultaneous investigation of multiple cell populations (local tissues, local fluids, blood) and quantification of numerous pharmacological and biological parameters (activation markers and a large variety of intracellular mediators). This approach is technically feasible using laboratory methods, since ethical considerations will obviously limit the number of NSAID provocations acceptable in hypersensitive patients.

Acknowledgments

MLS and PG are supported by grant RD07/0064 from the Spanish Research Network on Adverse Reactions to Allergens and Drugs (RIRAAF: Red de Investigación de Reacciones Adversas a Alérgenos y Fármacos) of the Carlos III Health Institute.

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Manuscript received January 17, 2009; accepted for publication June 3, 2009.

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