

PAF acether release on antigenic challenge

A method for the investigation of drug allergic reactions

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This study describes an *in vitro* method to evaluate a PAF acether release test (PART) from white blood cells after antigenic challenge. PAF acether activity of the supernatant was tested by platelet aggregation. The aggregating power was abolished by using SRI 63-441 (Sandoz), a PAF acether inhibitor. This method was applied to 57 patients with allergic or pseudo-allergic reactions to drugs by using different drug protein conjugates. The results of PART were evaluated in relation to the clinical history (score of imputability) and to other tests (skin tests, lymphocyte transformation tests (LTT), IgE-RAST). A good correlation was found between the release of PAF acether and a high predictability score: sensitivity 75%, specificity 83.8%. PART also correlated with skin tests (75% agreement, $n = 60$), with LTT (67.7% agreement, $n = 74$) and in 65.6% of cases with positive penicillin IgE-RAST ($n = 32$). This method brings a new possibility for the investigation of drug-allergic and pseudo-allergic reactions.

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The involvement of PAF acether in allergic reactions and in inflammatory processes is well established (2, 4, 6, 8, 17, 20, 24). This powerful mediator of inflammation can be released upon activation, by various stimuli, of different immune cells, such as basophils, macrophages or neutrophils (16). Particularly, this neoformed mediator can be released from sensitized basophils and mast cells upon antigenic challenge (3, 5, 23).

We were interested to find whether PAF acether could be released specifically *in vitro* by an antigen and if this could eventually be used as a new method for the investigation of allergic diseases. We therefore tested a method *in vitro* and applied it to the diagnosis of drug allergy using drugs linked to human serum albumin (HSA) as protein carrier. We report here the first results obtained by this approach and compared the results with those obtained by other classic methods used in the investigation of drug allergies (9, 13).

Material and methods

Patients and controls

The study comprised 57 patients from the Allergy Clinic of the University Hospital of Geneva, all of whom were strongly suspected of having drug allergy: 36 to penicillins, 8 to cotrimoxazole (trimethoprim-sulfamethoxazole) or one of its compo-

nents, 12 to aspirin, and 12 to lidocain. The patients were distributed into 2 groups: Group I had true allergic reactions by history (immediate or delayed hypersensitivity) and Group II had suspected pseudo-allergic, idiosyncratic or intolerance reactions (mainly patients with untoward reactions to aspirin or lidocain). The patients were allocated to each group according to a clinical predictability score for the suspected drug. This score is established using a questionnaire (19) taking into account, firstly the chronology of events (delay of appearance of symptoms, effect of discontinuing the treatment and of challenge with the drug) and, secondly, disease symptoms (suggestive history, favouring factors, other causes non-drug related). The results are scored from 0 (doubtful) to 6 (highly suggestive of an allergic or pseudo-allergic reaction to the suspected drug) (19).

PART was also applied to a control group (i.e. volunteer patients with similar drug treatment but without allergic reaction) to ensure that the different conjugates did not induce a non-specific release of PAF acether. Ten volunteers were recruited for each drug tested.

Skin tests with the suspected drug were performed in each patient, first by prick-test, using a 1 mg/ml solution and if negative, intradermal tests were done with serial dilutions starting at 10^{-3} mg/ml.

Lymphocyte transformation tests (LTT), which can

be used to demonstrate drug hypersensitivity (1, 13, 15, 21), were performed with the suspected drug using the method previously described by Girard et al. (12).

IgE-RAST were done with the sera of patients with suspected penicillin allergy, using commercially available penicillin nitrocellulose disk and RAST kits (Pharmacia® Uppsala, Sweden).

Preparation of hapten-protein conjugates

Coupling was made at basic pH (10.4 to 12) using human serum albumin (HSA) as protein carrier and drugs corresponding to the Pharmacopoeia Helvetica VI. To prepare penicilloyl HSA, lidocain HSA, aspirin HSA, cotrimoxazole HSA and sulfamethoxazole HSA conjugates, the drugs were first dissolved in phosphate buffered saline pH 7.4 (PBS), then added to a 10 mg/ml HSA solution. To prepare trimethoprim HSA conjugate, trimethoprim was dissolved in dioxan 1,4 then added to the same HSA solution. Each coupling was made with an excess of drug. After 18 h incubation, each conjugate was extensively dialysed against PBS to eliminate the solvent and the remaining free drug. Conjugates were stored at -18°C until use.

The amount of drug conjugated was assessed by fluorescence polarization immuno-assay (F.P.I.A., Abbott TDX®, Abbott Diagnostics®, IL) for the drugs for which this assay was available. The degree of conjugation was reproducible from batch to batch for each given drug ($<20\%$ variation), i.e. aspirin: 1.59 ± 0.24 mol/mol, lidocaine: 45.5 ± 9.0 mmol/mol.

PAF acether release test

Release of PAF acether in vitro. Heparinized blood (50 IU/ml whole blood) was collected from the allergic patient. White cells were separated on Ficoll-metrizoate (metrizoic acid sodium salt (Sigma®) 75%:Ficoll 5.6% (Pharmacia®), 1:6.9 v/v), then washed in a Tris saline buffer (Tris-HCl 10 mM, NaCl 136 mM, pH 7.4) (23) and centrifuged for 10 min at 1200 rpm. After 2 washings, cells were counted, suspended in tyrode albumin buffer at pH 10.4 (7). Drug protein conjugate was then added and the volume of the suspension adjusted with tyrode albumin buffer to obtain a 1.10^7 cells/ml final suspension, according to the method described by Tence et al. (23). Several dilutions of hapten protein conjugate were used. The cell suspension was incubated overnight at 37°C . Supernatant, with or without PAF acether, was then recovered and stored at -18°C until use. To control the absence of PAF acether release in the experimental conditions, the cells of patients were also incubated with the buffer

alone. Cellular viability in the experimental conditions was ensured by using an exclusion test with Trypan blue. Moreover, to ensure that the drug conjugates did not induce a non-specific release of PAF acether, each of them was tested with healthy donors' cells, and with cells from allergic patients presenting no reaction to the substance under test.

Platelet aggregation test

Venous blood was taken with sodium citrate (3.8% 1:10 v/v) from a healthy donor then centrifuged at room temperature at 800 rpm for 15 min. The platelet-rich plasma (PRP) was recovered and the pellet centrifuged once more at 4000 rpm for 10 min to obtain platelet poor plasma (PPP). PAF acether activity of the supernatant was tested by measuring the aggregation of PRP induced, using an aggregometer (PAP 4, Bio Data Corporation).

200 μl of PRP was introduced in an aggregometer tube under stirring at 1100 rpm. 20 to 60 μl of supernatant was added to the tube and changes in light transmission were recorded. PAF acether activity of the supernatant was evaluated in percent of aggregation induced by a synthetic PAF acether solution (L- α -phosphatidylcholine, β -acetyl- γ -O-(octadec-9-cis-enyl) (Sigma)), solution 1 μM in tyrode albumin buffer used as positive control.

Use of PAF acether inhibitor = SRI 63-441

To ensure that the aggregating agent released was in fact PAF acether, a PAF acether inhibitor was used: SRI 63-441 (14), (cis(+/-)-1-[2[hydroxy[[tetrahydro-5[(octadecylaminocarbonyl)oxy]methyl] furan-2-yl]methoxyphosphinyloxy]ethyl]-quinolinium hydroxide) kindly provided by Doctor J. Morley (Sandoz, Basel, Switzerland). When a supernatant exhibited an aggregating power, the platelet aggregation test was repeated in the presence of the inhibitor: successively, 20 μl of the inhibitor solution then the aggregating supernatant were added to the platelet suspension and aggregation inhibition recorded. 0.1 μM , 1 μM , 10 μM and 100 μM solutions of SRI 63-441 in Tris tyrode buffer (CaCl₂.6 H₂O 100 mM: 0.65 ml; MgCl₂.6 H₂O 100 mM: 1.22 ml; NaCl: 8 g; Tris: 1.21 g; HSA: 250 mg; qsp distilled H₂O 100 ml) have been used. The inhibiting capacity of aggregation was also treated with the following agents: ADP 10 μM , arachidonic acid 5 mg/ml, PAF acether 1 μM .

Statistical analysis

The distribution between release and non-release of PAF acether versus clinical score was analysed with a Mann-Whitney test. Results obtained with PART

Table 1. Relation between release or non-release of PAF acether *in vitro* and the clinical score. Statistical analysis using a Mann-Whitney test shows a significant difference between the 2 distributions with $p < 1.10^{-6}$

		Score								
Drug		C	0	1	2	3	4	5	6	
PAF release	Penicillin					3	5	4		
	Aspirin					2	1			
	Cotrimoxazole and/or separate components					1	1	1		
	Lidocain					1				
	Total (n=19)					7	7	5		
No PAF release	Penicillin	10	2	1	4	14	3			
	Aspirin	10	1	1	4	3				
	Cotrimoxazole and separate components	10	~	1	1	2	1			
	Lidocain	10	2	2	4	3				
	Total (n=49)		5	5	13	22	4			

C=control population.

were compared with those obtained by LTT and by skin test. The discordant responses (i.e. for the same patient, positive results by using one method and negative by using the PAF acether release method or *vice versa*) were analysed by a McNemar test to find whether distribution of the differences was symmetric.

Results

PART and clinical score. A good correlation was observed between the release of PAF acether and a high clinical score (score > 3), whereas the absence of PAF acether release correlates with a low clinical score (Table 1). Statistical analysis using a Mann-Whitney test shows a significant difference between the 2 distributions with $p < 1.10^{-6}$. A release of PAF acether was never observed among negative controls.

When considering a score ≥ 4 as indicative of an allergic reaction, the sensitivity of the test was found to be 75% and the specificity 86.5% (Table 2). The predictive values were respectively of 63.1% for the positive tests and 76.2% for the negative tests, with an efficiency of 83.8% (Table 2).

PART and LTT. When comparing the results obtained with PART and LTT, a good agreement could be found in 67.6% of the tests ($n = 74$) (Table 3). The discrepancies between the release of PAF acether without lymphoblastic stimulation and non-release of PAF acether and cellular proliferation were analysed by a McNemar test and did not show a non-symmetric distribution of differences ($\alpha = 5\%$).

PART and skin tests. Because of skin dermatographism or for clinical reasons (risk of anaphylac-

Table 2. Evaluation of the PAF acether release method

	Release of PAF acether	No release of PAF acether
Score ≥ 4	A = 12	B = 4
Score < 4	C = 7	D = 45
Predictive value, positive	$\frac{A}{A + C} = 63.1\%$	
Predictive value, negative	$\frac{D}{B + D} = 76.27\%$	
Sensitivity	$\frac{A}{A + B} = 75\%$	
Specificity	$\frac{D}{C + D} = 86.53\%$	
Efficiency	$\frac{A + D}{A + B + C + D} = 83.82\%$	

Table 3. Comparison of the results obtained with lymphoblastic transformation tests (LTT) and PAF acether release (PART)

Drug		No. patients	Positive LTT	Negative LTT
PAF release	Penicillin	12	5	7
	Aspirin	3	0	3
	Cotrimoxazole	2	2	0
	Trimethoprim	0	0	0
	Sulfamethoxazole	0	0	0
	Lidocain	1	0	1
	Total	18	7	11
No PAF release	Penicillin	23	5	18
	Aspirin	13	3	10
	Cotrimoxazole	3	1	2
	Trimethoprim	3	0	3
	Sulfamethoxazole	4	1	3
	Lidocain	10	3	7
	Total	56	13	43

tic shock), only the results of 60 skin tests to different drugs were available (Table 4). The results between PART and skin tests agreed in 75% of the tests and the divergences were equally distributed (Table 4).

PART and IgE-RAST. Among the patients with a suspected penicillin allergy, 32 RAST to penicillin were performed. A positive correlation was observed in 65.6% of the cases. The divergences were also equally distributed.

Inhibition assay using SRI 63-441. The results obtained with the PAF acether inhibitor SRI 63-441 are shown in Table 5. Final concentration of the inhibitor varying from 0.1 μM to 1.0 μM abolished the aggregating activity of an 1 μM PAF acether solution or the aggregating activity of the supernatant recovered. However, the same concentration of the inhibitor was without effect on the aggregating power

Table 4. Comparison of the results obtained with skin tests and PAF acether release

	Drug	Positive skin tests	Negative skin tests
PAF release	Penicillin	6	3
	Aspirin	0	1
	Cotrimoxazole	3	0
	Trimethoprim	0	1
	Sulfamethoxazole	1	0
	Lidocain	0	0
	Total	10	5
No PAF release	Penicillin	3	17
	Aspirin	2	4
	Cotrimoxazole	3	2
	Trimethoprim	1	2
	Sulfamethoxazole	1	2
	Lidocain	0	8
	Total	10	35

Table 5. Inhibiting power of SRI 63-441

[SRI 63-441] μ M	PAF 1 μ M	Arachidonic acid 5 mg/ml	ADP 10 μ M
0.01	-	-	-
0.1	-	-	-
1	-	-	-
2	Partial	-	-
5	+	-	-
10	+	Partial	Partial
100	+	+	+

- = no inhibition of the aggregation induced by the agonist.

+ = inhibition of the aggregation induced by the agonist.

of a 5 ml/mg arachidonic acid solution and a 10 μ M ADP solution. To neutralize the aggregating activity of arachidonic acid or ADP, the final concentration of SRI 63-441 had to be higher than 10 μ M.

Discussion

This study demonstrates that *in vitro* incubation of peripheral white cells from an allergic patient with a drug protein conjugate can result in their activation and the release of mediator. The use of a specific inhibitor (SRI 63-441) and the ensuing suppression of the aggregating activity of the recovered supernatant by a high dilution of this inhibitor, strongly suggest that the mediator released is PAF acether. PAF acether is a neoformed mediator (23, 24), its release upon the influence of drug antigen in experimental conditions must be specific. Several controls were carried out and these confirm this assertion. Release of this mediator by drug antigen in a non-allergic subject was not observed, and the incubation buffer alone (tyrode albumin buffer) without antigen failed to induce a release of PAF acether in allergic patients. Lastly, in a patient with allergic reaction to

a given drug, the incubation of his cells with another drug conjugate did not produce PAF release.

In preliminary tests performed by using the drug alone (1 mg/ml), a significant release of PAF acether could be obtained only in isolated tests (results not shown). This observation would agree with the general hypothesis that in order to become antigenic, most drugs which are low molecular weight compounds, must be coupled to a carrier (18). Several authors have already observed a better response in skin tests (11) and in LTT (13) by using hapten-protein conjugates instead of the hapten alone. However, in this preliminary work, only one hapten-protein conjugate has been tested for each drug and it would be interesting to know if the efficiency of this test can be improved by modifying the hapten/carrier ratio and the accessibility of the hapten on the carrier. Experiments are in progress to show whether PAF acether release could also be obtained with free drugs using different concentrations.

The clinical score established from the clinical history represents a reasonable approach to evaluate this method, and the statistical analysis of the results obtained shows a good reliability between the release (or non-release) of PAF acether and this clinical score.

No false positive results could be observed. There were some cases in which we could not observe a release of PAF acether in spite of a high clinical score. This concerns 3 cases of suspected allergy to penicillin and one case of suspected allergy to cotrimoxazole also for which neither skin tests, RAST or LTT could confirm the clinical impression. Thus, it appears that in these 4 cases, evaluation with the clinical score was overestimated, or that the proper antigen was not investigated, i.e. an antigenic metabolite, an additive or a preservative was overlooked.

The specificity, sensitivity and efficiency of the method are satisfactory. However, if the predictive value for the negative tests is fairly good (76%), the predictive value for the positive tests is only of 63%. Further experiments are required to improve this performance. The results obtained were compared with other tests commonly used in our department. We could show that the skin tests and the LTT were in good agreement with the release of PAF and that the discordant results are equally distributed.

When PART and RAST were compared for penicillin, the two tests gave only 65.6% concordant results. The RAST estimates the circulating level of IgE antibodies specific to penicillin, whereas the PAF test is related to the cellular response of sensitized white blood cells upon antigenic challenge. It has already been shown that humoral sensitization to penicillin is not automatically related to a hypersensitivity reaction upon re-exposure to a β -lactam

antibiotic (10). This may in part explain the discordant results observed between RAST and PART.

The first analysis of clinical elements shows that PAF acether can be released in various types of physiopathological manifestations. A release of PAF acether was obtained in patients with intolerance to aspirin, which is usually non-immunologically mediated. Aspirin and other non-steroid anti-inflammatory drugs inhibit the cyclooxygenase pathway, which can lead via the lipoxygenase pathway to the formation of large amounts of leukotrienes (18, 22). However, PAF biosynthesis is independent of the cyclooxygenase (4), so this result suggests that the release of PAF acether upon aspirin HSA challenge is mediated by different mechanisms that still need to be elucidated.

At this stage, it is not yet possible to relate the release of PAF acether upon antigenic challenge *in vitro* with a precise mechanism. However, there is no universal test that can establish with certainty the diagnosis of allergy or pseudo-allergy to drugs. Thus, it is usually necessary to use several tests to confirm or exclude the involvement of a drug in an adverse reaction. The patients in which PAF acether release was observed *in vitro* exhibited immediate type as well as semi-delayed or delayed type allergic reactions. PART could be used as a new tool for the investigation of allergic diseases and, more particularly, for the investigation of drug allergy. The procedure is easy and fast. Because PAF acether is neoformed, PART is also highly specific and could be used as a new *in vitro* "effector" test.

Further experiments are needed to establish more precisely the immunopathological mechanisms involved in the actual release of PAF acether.

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