Asian Pacific Journal of Allergy and Immunology (1992) 10 : 25-32

The Presence of Specific IgE to Salicyloyl and O-Methylsalicyloyl in Aspirin-Sensitive Patients

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SUMMARY **Certain adverse reactions to aspirin (ASA), nonsteroidal anti-inflammatory drugs (NSAIDs) and pyrazoline derivatives resemble IgE-mediated hypersensitivity. However, convincing evidence of antigen-antibody interactions or of the inhibition of the cyclooxygenase pathway of arachidonic acid metabolism leading to an increase in the generation of leukotrienes (LTs) and a decrease in the generation of prostaglandins (PGs) was not fully demonstrated. In this study, two types of specific IgE antibodies have been found in 27 serum samples from 28 ASA-sensitive patients with salicyloyl-discs and O-methylsalicyloyl-discs by Radio Allergo Sorbent Tests (RAST). The positive rates were 96.4% and 71.4%, respectively. In contrast, no positive results could be found in 10 normal donors without ASA sensitivity after ingestion of ASA 500 mg/day for 14 days. Further investigation of the chemical structure of epitopes was done by cross inhibition studies. Our results are an increasing evidence in favour of an IgE-dependent mechanism in patients suffering from ASA sensitivity. Hopefully, the determination of specific IgE antibodies will be a safe diagnostic method of ASA sensitivity** *in vitro*.

Over the past two decades, considerable progress in the field of ASA sensitivity has been made in knowledge of immunopharmacology, biochemistry, clinial diagnosis, cross reactions with NSAIDs and desensitization.[1-7] Nevertheless, the pathogenesis still remains obscure[8] and no safe diagnostic method has been established without exposing the patients to the risk of taking ASA.[9,10] In view of the clinical symptoms of ASA sensitivity resembling IgE-mediated hypersensitivity, mainly including generalized urticaria/angioedema, bronchial asthma, rhinoconjunctivitis, a number of immunological mechanisms have been investigated.

In 1967 Minden[11] first reported that the sera of patients receiving ASA contained antibodies which reacted with acetylated-human serum albumin (HSA), but the presence of these antibodies did not correlate with clinical sensitivity to ASA. Later, de Weck[2] discovered that a contaminant of commercial ASA preparation, aspirin anhydride, was a highly immunogenic substance probably inducing the formation of aspiryl-specific IgE antibodies in patients ingesting ASA. However, only three patients sensitive to ASA with urticaria showed positive skin tests to aspiryl-polylysine (PLL). In addition, Phells[12] reported positive skin tests to aspiryl-PLL in six patients with urticaria sensitive to ASA. In contrast, Weltman[13] reported that no aspiryl-specific IgE antibodies were detected in 30 serum samples from ASA-sensitive patients. Likewise, Schlumberger[3] investigated 17 ASA-sensitive patients, none of them had positive skin tests to N-salicyloyl- and acetylsalicyloyl-PLL. Furthermore, the sera of these patients were transferred to rhesus monkeys which then were challenged by intravenous or intradermal injection of N-salicyloyl-bovine serum albumin or N-acetyl-salicyloyl-PLL or by ingestion of aspirin anhydride or ASA; but no positive reaction was obtained.

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Aside from immunological research, in 1971 Vane[14] first discovered a common pharmacological effect shared by ASA and NSAIDs, both of them interfered with the metabolism of arachidonic acid via inhibition of the cyclooxygenase, preventing the generation of prostaglandin E_2, D_2 $F_{2\alpha}$ (PGE_2, D_2, $F_{2\alpha}$). The PGE_2 effects bronchodilation, while PGD_2 and $PGF_{2\alpha}$ enhances bronchoconstrictor tonus. Thus, it was postulated by Szczeklik[4] that in susceptible individuals inhibition was predominantly directed toward the PGE_2. Parker[15] and Burka[16] then suggested that if the cyclooxygenase pathway of arachidonic acid metabolism was blocked by ASA or ASA-like drugs, the reaction proceeded along the lipooxygenase pathway releasing the increased amounts of LTs, primary mediators leading to asthmatic attack. Hence the theory of 'shunting'. By contrast, in 1976 Stevenson[5] reported that asthmatic reactions to ASA were associated with release of histamine into plasma in the asthmatic patients with ASA-sensitive patients, and a recent study by Ferreri[7] demonstrated that increased levels of histamine and LTC_4 could also be detected in nasal secretions of ASA-sensitive patients with naso-ocular and bronchospastic symptoms during reactions to ASA with relatively low provoking dose (150 mg), whereas levels of PGE_2 decreased significantly in nasal secretions of normal donors after ingestion 650 mg of ASA and these decreases were not associated with histamine release or increased LTC_4.

In the present study, we investigated 28 serum samples from ASA-sensitive patients. Evidence is shown that specific IgE antibodies are able to be detected with salicyloyl-discs and O-methylsalicyloyl-discs by RAST. The possibility of an IgE-dependent mechanism in ASA sensitivity, an analysis of the relation between specifc IgE antibodies and clinical symptoms and an evaluation relevant to the use of RAST as a clinical diagnostic method are discussed.

MATERIALS AND METHODS

Serum samples and patients' conditions

Sera from 28 patients (12 males and 16 females) who had previous positive histories of ASA sensitivity were taken out after last ASA sensitivity or positive challenge tests. Their ages ranged from 20 to 81 years, with a mean of 41 years. Twenty-two patients were documented by oral ASA challenges in our clinic according to a previously described method.[5,17] The ASA doses for a positive challenge had a very wide range from 25 to 500 mg. The clinical symptoms included asthma (10) (1 with generalized flushing and pruritis), urticaria/angioedema (16), generalized flushing with pruritis (1) and rhino-conjunctivitis (1), all of which happened within 4 hours after challenges. Before ASA sensitivity was discovered, 17 patients, except patient No. 14, had atopic states basically according with symptoms of ASA challenges. Total IgE concentration was determined by Phadebas IgE RAST (Pharmacia Diagnostics AB, Uppsala, Sweden). Fifteen out of 27 samples had total IgE concentrations more than 100 IU/ml. ASA-intracutaneous tests were performed in 18 patients by injecting 0.02 ml of a 0.1% solution of the drug, but only 2 showed weak positive results (Table 1). Another 10 serum samples were obtained from normal donors who had taken ASA without sensitive reaction. They were given ASA 500 mg/day for 14 days again before blood sampled. A serum pool from blood samples of 100 healthy students with total IgE of 51 IU/ml was prepared for control purpose.

Reagents

Monoclonal anti-human IgE (BS 17) and Amino-spacer discs were kind gifts from Dr BM Stadler and Dr M Walti (Bern, Switzerland). Sodium iodide (I^{125}) was obtained from Amersham Buchler GmbH & Co. KG (Braunschweig, Germany). Discs (round filter ϕ 6 mm No. 311 660) were supplied by Schleicher & Schuell Co. (Dassel, Germany). The method of I^{125}-labelling of revealing-antibodies for RAST is described elsewhere.[18] All the other chemicals were of analytic grade purchased from Fluka AG (Buch, Switzerland) and Sigma Chemical Co. (St. Louis, USA).

Preparation of salicyloyl-O-succinimide and O-methylsalicyloyl-4-nitrophenyl ester

Salicylic acid (2.7 g) was solubilized in 100 ml of tetrahydrofuran. N,N-dicyclohexylcarbodiimide (4.12 g) and N-hydroxysuccinimide (2.6 g) was then added and stirred for 24 hours. The suspension was filtered and the filtration diluted with 500 ml of dichloromethane was poured into a separating funnel, then washed with 500 ml of 2.5% potassium carbonate. After the dichloromethane-phase was collected and washed again with distilled water, the sodium sulfate was put in until it became clear, which then was filtered. The filtration was evaporated by rotary evaporator under vacuum at 40°C until white powder-like residue yielded. The white product (salicyloyl-O-succinimide ester) with melting point of 145-146° C was kept in a desiccator using negative pressure and desiccant to dry for overnight.

O-methylsalicylic acid (1.52 g) was solubilized in 50 ml of ethylacetate. N,N-dicyclohexylcarbodiimide (2.1 g) and 4-nitrophenol (1.4 g) were then added and stirred for 24 hours. The suspension was filtered and the filtration diluted with 500 ml of dichloromethance was poured into a separating funnel, subsequently washed with 1,000 ml of 0. 1N HCL, 500 ml of distilled water, 100 ml of 2.5% potassium carbonate and distilled water. The following steps were the same as the preparation of salicyloyl-

Table 1. Personal, clinical, oral challenge and RAST data from 28 individuals of reactions to ASA by history

Pat No.	Age (yr)	Sex	Total IgE (IU/ml)	Atopic states	Reactions of ASA	Time	Skin tests IC	ASA doses of challenge (mg)	Results of RAST SA	Results of RAST OMS
1	25	F	65	/	A	2 yr	ND	ND	+	+
2	25	M	385	A, S	A	5 yr	–	150	+	+
3	65	F	75	/	A	1 yr	ND	ND	+	–
4	36	F	136	A, R	A	?	ND	ND	+	+
5	33	F	106	/	A	4 yr	ND	ND	+	+
6	52	M	99	A, R, S, FA	A	5 yr	–	25	+	+
7	67	F	124	A*	A	5 yr	ND	500	+	–
8	42	F	71	/	A	5 yr	–	ND	+	–
9	22	M	332	/	A,GFP	7 da	–	25	+	+
10	55	M	1,000	A	A	2 yr	–	50	+	+
11	66	M	12	CU	U,Q	3 yr	–	50	+	+
12	46	M	31	CU	U	7 yr	–	500	+	+
13	34	F	63	CU	U	2 yr	–	50	+	+
14	29	M	1,000	A,R	U	1 mo	ND	500	+	–
15	44	F	267	AU	U	3 yr	ND	400	–	–
16	54	M	27	CU	Q	1 yr	ND	500	+	+
17	38	M	32	/	U Q	3 yr	–	150	+	+
18	81	F	?	/	U	7 d	–	ND	+	–
19	37	F	147	CU,Q	UQ	5 mo	–	200	+	–
20	28	M	32	CU	U	3 yr	+	500	+	+
21	55	F	13	CU,Q	U	41 yr	–	25	+	+
22	20	F	160	/	Q	1 yr	+	125	+	+
23	30	M	194	CU	U	7 d	–	25	+	+
24	26	F	159	/	U	1 mo	–	500	+	+
25	34	F	194	/	U,Q,C	4 mo	–	50	+	+
26	43	F	271	/	U	14 d	–	100	+	+
27	27	F	31	CU	GFP	5 mo	ND	50	+	+
28	28	M	100	/	R,C	1 yr	–	500	+	–

Pat = patient number; Time = the period elapsed between beginning ASA sensitivity to the blood taken out in this study; IC = intracutaneous tests with 0.1% ASA; SA = salicyloyl-discs, OMS = O-methylsalicyloyl-discs; / = no, ND = not done; + = positive, – = negative; A/A* = intrinsic/ extrinsic asthma, S = sinusitis, R = rhinitis, C = conjunctivitis, A/CU = acute/chronic urticaria, Q = angioedema, GFP = generalized flushing with pruritis, FA = food allergy.

O-sucinimide ester. The final product was O-methylsalicyloyl-4-nitrophenyl ester with melting point of 76-77° C.

Preparation of salicyloyl-discs and O-methylsalicyloyl-discs

Amino-spacer discs (300 mg) were added to a solution including salicyloyl-O-succinimide ester or O-methylsalicyloyl-4-nitrophenyl ester (5 mg) in 20 ml of 0.5 M $NaHCO_3$, pH 8.4. The suspension was rotated end-over-end at 4°C for 24 hours. Afterwards, the supernatant was aspirated and the discs were washed with 20 ml of 0.5 M $NaHCO_3$, pH 8.4. The remaining reactive groups on the discs were blocked with 20 ml of 0.05 M ethanolamine in 0.1 M $NaHCO_3$ at room temperature for 2 hours. This was followed by washings with distilled water, 0.1 M $NaHCO_3$, pH 8.4, 0.5 M $NaHCO_3$, pH 8.4 and then resuspended in 0.1 M phosphate-buffered saline (PBS), pH 7.5 containing 0.5% HSA and 0.02% sodium azide. The discs were stored at 4° C until use (Figs. 1, 2).

RAST procedure

Fifty microlitres of serum were placed onto each disc in plastic tubes

(No. 55. 484, Numbrecht, Germany) in duplicate. All these tubes were then covered and incubated at room temperature for 3 hours. Two millilitres of 0.9% NaCl containing 0.2% Tween 20 were added to all tubes for 10 minutes. This washing procedure was repeated twice. A 50 μl aliquot of monoclonal anti-human (I^{125}) IgE solution was dispensed onto each disc and the tube re-incubated for overnight. The discs were then washed as before and placed into new tubes. Radioactivity in all tubes and duplicate tubes containing 50 μl of monoclonal anti-human (I^{125}) IgE was measured in a γ-counter (Berthold, Isernhagen, Germany) for 1 minute and results were expressed as counts per minute (cpm). At least three control serum samples were tested in each assay and the mean value of cpm + 3 standard deviations (mean CS + 3 SD) was calculated. Tested sample values above this were considered positive.

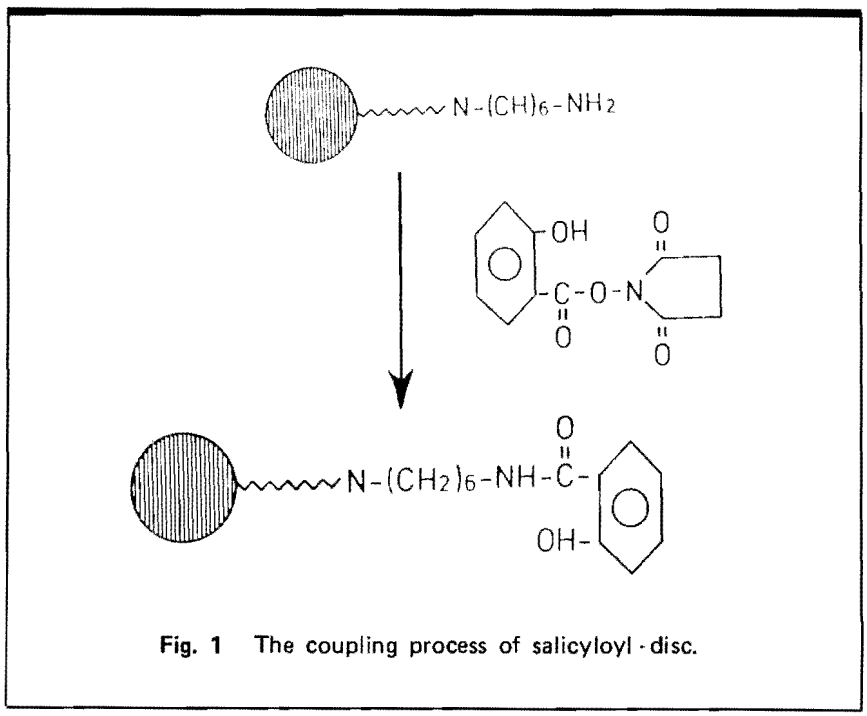

Fig. 1 The coupling process of salicyloyl-disc.

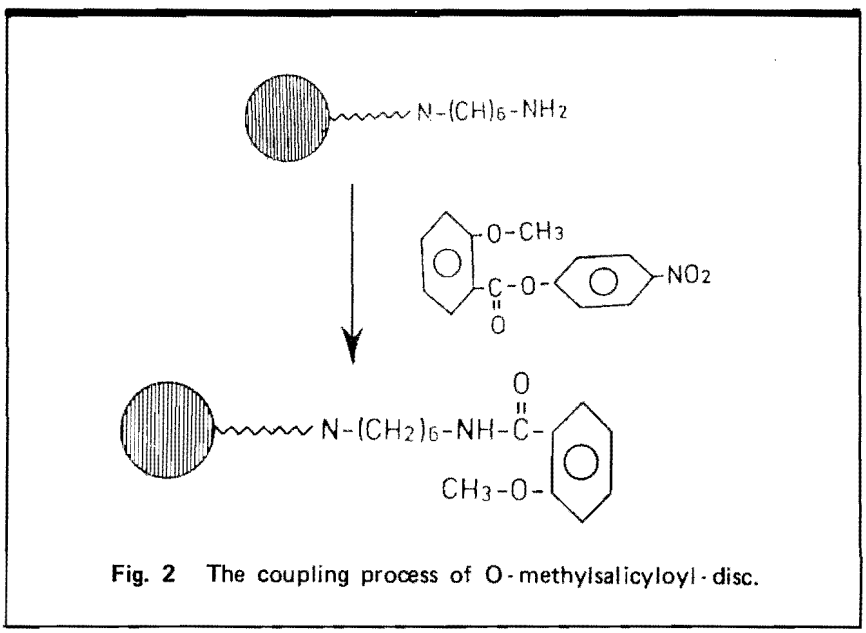

Fig. 2 The coupling process of O-methylsalicyloyl-disc.

RAST inhibition assay

Briefly, 50 μl of tested serum was incubated with 50 μl of either chemicals in distilled water (+che) or just distilled water (-che) at room temperature for 1 hour. After centrifugation at 3,000 rpm for 15 minutes 50 μl were taken out and tested by RAST procedure on the relevant disc. Percentage of inhibition values were calculated using the formula:

$$\text{Percentage of inhibition} = 100 - \frac{\text{cpm of (+che)} - \text{cpm of control serum}}{\text{cpm of (-che)} - \text{cpm of control serum}} \times 100$$

RESULTS

RAST results are shown in Table 1 and Fig. 3. Comparing 10 serum samples from normal donors after taking ASA 500 mg/day for 14 days with control serum by using salicyloyl-discs, I^{125} uptake of the treated donors' samples was under the value of man CS + 3 SD. In contrast, 27 out of 28 serum samples from ASA-sensitive patients compared with control serum showed values more than the mean CS + 3 SD; only one sample (No. 15) from the patient with urticaria after oral challenge had a value less than the mean CS + 3 SD. Likewise, the I^{125} uptake of serum samples from the same donors compared with control serum by using O-methylsalicyloyl-discs showed values within the mean CS + 3 SD range, while 20 serum samples of the same ASA-sensitive patients gave positive results. Other negative samples were obtained from patients suffering from ASA sensitivity with symptoms of asthma (3) urticaria/angioedema (4) and rhino-conjunctivitis (1). However, these was no

significant difference of I^{125} uptake on salicyloyl-discs and O-methylsalicyloyl-discs in the group of patients with asthmatic symptoms (No. 1-10) having the mean values of +44.2% and +34%, respectively ($p > 0.05$); there was also no difference in the group of patients with urticaria/angioedema (No.11-26) counting +58.64% in average and +35.76% respectively ($p > 0.05$). The RAST results showed a moderate correlation ($r = 0.76$) between these two types of discs (Fig. 4).

To investigate the specificities of IgE antibodies, further data were obtained from inhibition studies. The serum sample of No. 9 was used and incubated with salicylic acid, 2-aminophenol, 4-aminoantipyrine, O-methylsalicylic acid, ASA and indomethacin, then tested with salicyloyl-discs and O-methylsalicyloyl-discs by RAST as described before. Interestingly, the salicylic acid and 2-aminophenol had positive inhibition results (> 50% inhibition) on salicyloyl-discs, whereas 4-aminoantipyrine, O-methylsalicylic acid, indomethacin and ASA gave negative inhibition results. Likewise, O-methylsalicylic acid and indomethacin had positive inhibition results on O-methylsalicyloyl-discs, whereas others gave negative inhibition results (Fig. 5, 6).

To ascertain whether the total IgE would effect the results, we used two serum samples from atopic patients without ASA sensitivity having total IgE of 560 IU/ml and 1,748 IU/ml in comparison with control serum containing total IgE of 51 IU/ml on the two types of discs. The percentage change of I^{125} uptake ranged within ± 10%.

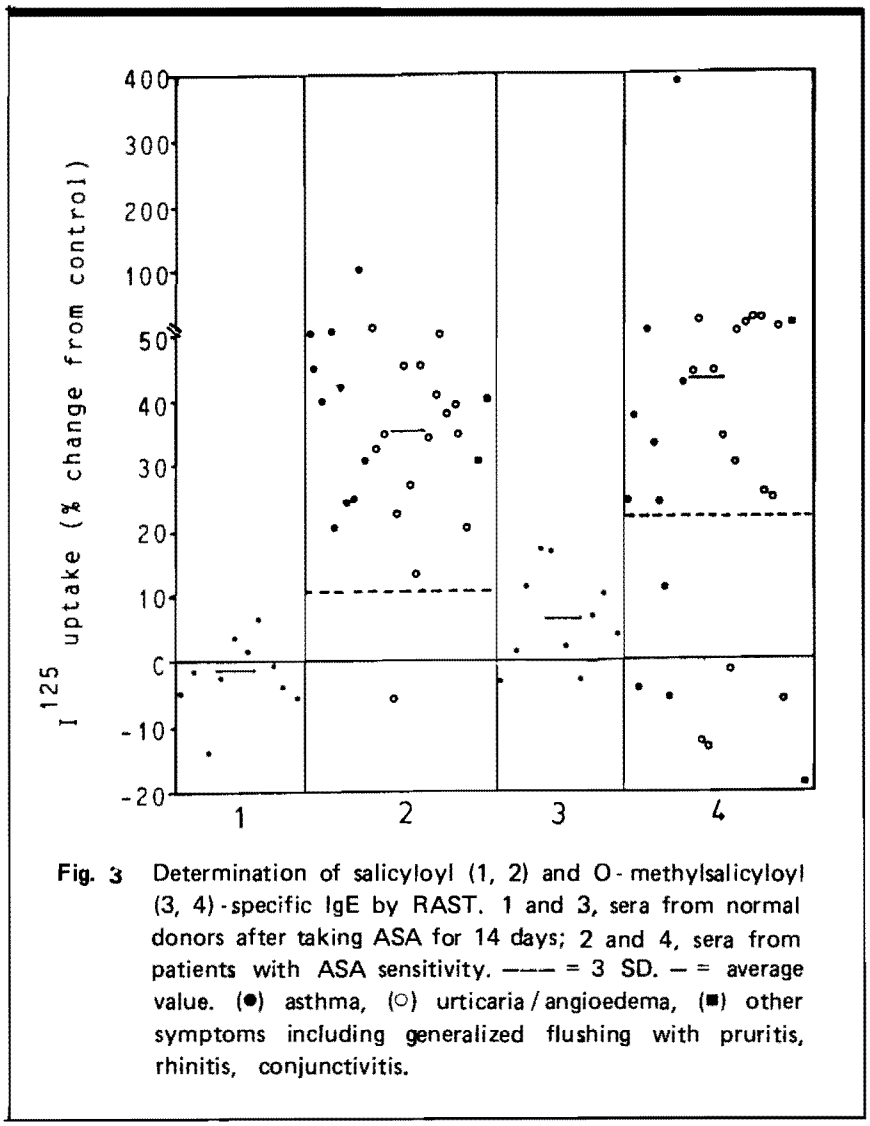

Fig. 3 Determination of salicyloyl (1, 2) and O-methylsalicyloyl (3, 4)-specific IgE by RAST. 1 and 3, sera from normal donors after taking ASA for 14 days; 2 and 4, sera from patients with ASA sensitivity. —— = 3 SD. — = average value. (●) asthma, (○) urticaria/angioedema, (■) other symptoms including generalized flushing with pruritis, rhinitis, conjunctivitis.

DISCUSSION

Our study demonstrated the presence of salicyloyl- and O-methylsalicyloyl-specific IgE antibodies by RAST in 96.4% and 71.4% respectively in 28 ASA-sensitive patients. Up to now, we only have knowledge of the metabolism of ASA in normal donors.[19] ASA is rapidly hydrolyzed to salicylic acid in plasma, liver and erythrocytes, of which 80-90% is bound to plasma proteins, especially albumin.[20] The positive inhibition tests with salicylic acid and 2-aminophenol on salicyloyl-discs showed the ortho-hydroxyphenyl group to be the epitope of salicyloyl-HSA and confirmed the existence of specific IgE antibodies against the epitope. However, the O-methylsalicylic acid, which is a derivative of ASA, could not be found in normal donors,[19] but specific IgE antibodies have been discovered by RAST in the group of ASA-sensitive patients. In addition, the previous reports by Szczeklik[4] that all his 11 ASA-sensitive asthmatics challenged with very low doses (1 to 25 mg) of indomethacin showed positive reactions, by Samter[21] that 18 ASA-sensitive asthmatics reacted with asthmatic symptoms to therapeutic doses of indomethacin on the first exposure, and by Pleskow[6] that cross desensitization with ASA and indomethacin could be established, indicated the possibility of similar chemical groups existing between O-methylsalicylic acid and indomethacin. In fact, indomethacin is a methylated indol derivative, which is 90% bound to plasma proteins,

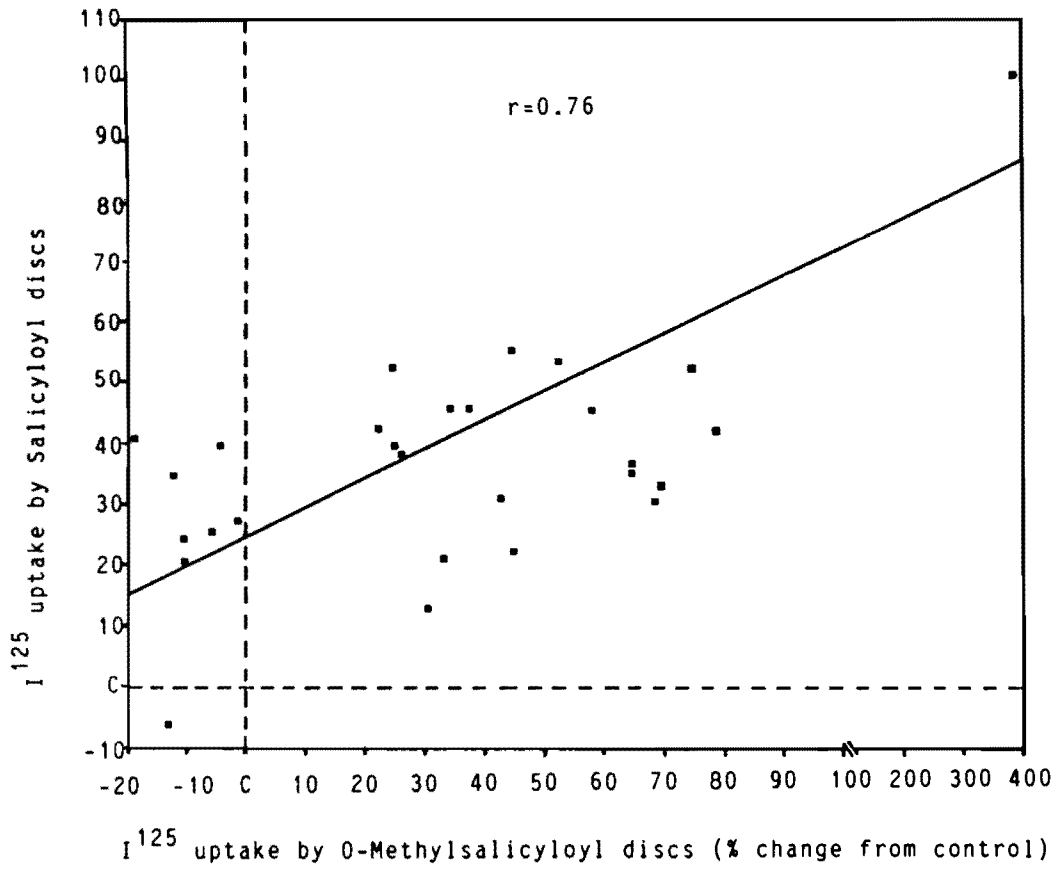

Fig. 4 Correlation between salicyloyl and O-methylsalicyloyl -specific IgE in sera from patients with ASA sensitivity.

and a methoxyphenyl group in the left part of indomethacin basically resembles an O-methylphenol group in the O-methylsalicylic acid.[20] Thus, both indomethacin and O-methylsalicylic acid probably represent similar epitopes, responsible for their clinical cross-reactions, cross-desensitizations and cross-inhibition tests. Moreover, some reports[22-26] on ASA sensitivity in siblings or family suggesting a genetic factor (socalled 'inborn errors of metabolism') combined with immunological background should be considered as underlying mechanism, which means that the ASA sensitivity is related to an abnormal methylation via O-methyltransferase transferring a methyl group to an ortho-hydroxy group of salicyloyl-HSA to be O-methylsalicyloyl-HSA, an antigen. The speculation is in accordance with a general rule of drug metabolism of 'phenyl group' substituents on the oxidation and methylation mainly occurring in the ortho-position of phenyl or phenol groups. Whether ASA has greater ability to activate the O-methyltransferase than salicylates and whether the dose-dependence of ASA challenge may be related to the ability of errors of metabolism is not know at the present time.

In view of the speculation drawn above and previous reports on most of ASA-sensitive patients, who were neither sensitive to salicylates nor showing positive skin tests to N-salicyloyl-PLL,[1,3,27] it should be considered that detected O-methylsalicyloyl-specific IgE may play a greater role in the pathogenesis of ASA sensitivity than salicyloyl-specific IgE.

According to the data of no significant difference between clinical symptoms (asthma or urticaria) and specific IgE antibodies, as well as the previous reports that 10-29% of ASA-sensitive patients with asthmatic attacks combining urticaria or angioedema,[3,28,29] the different clinical

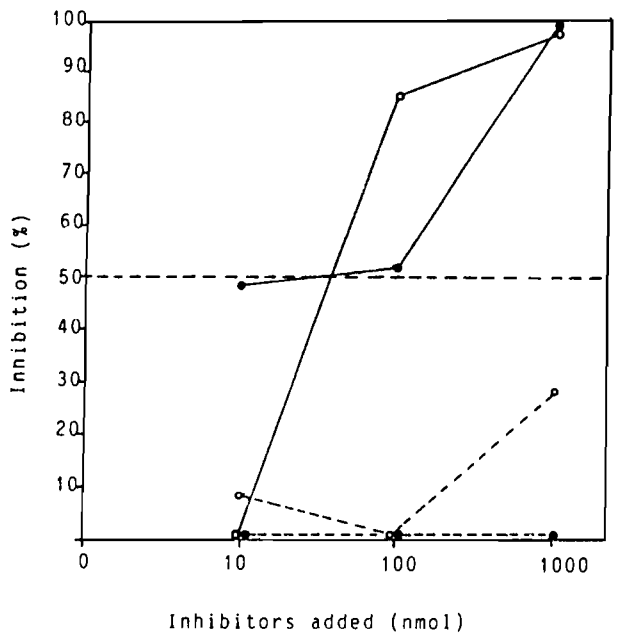

Fig. 5 RAST inhibition assays for salicyloyl-discs. Inhibitor: (●—) salicylic acid, (○—) 2-aminophenol, (○——) 4-aminoantipyrine, (●——) indomethacin, O-methylsalicylic acid and ASA. —— = 50% inhibition.

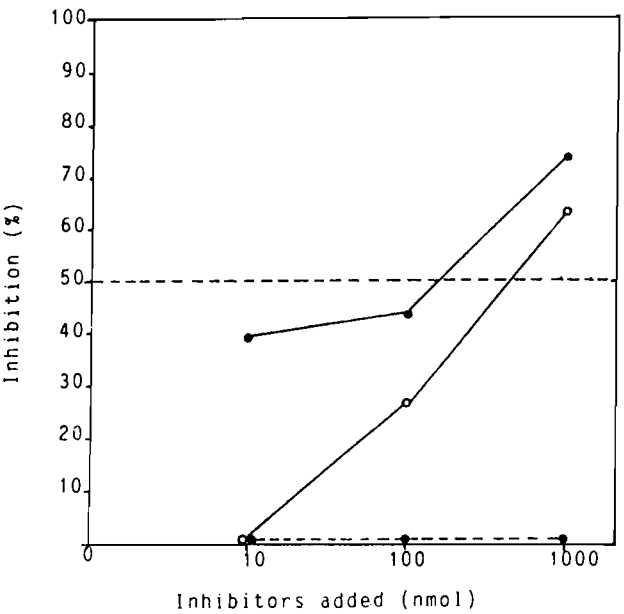

Fig. 6 RAST inhibition assays for O-methylsalicyloyl-discs. Inhibitors: (●—) O-methylsalicylic acid, (○—) indomethacin, (●——) ASA, 4-aminoantipyrine, salicylic acid and 2-aminophenol. ——=50% inhibition.

symptoms are probably due to the sensitized mast cells distributed differently in the organ systems of individuals.

In conclusion, two types of specific IgE antibodies have been discovered by RAST in ASA-sensitive patients; their epitopes were confirmed by cross inhibition studies. The possible pathogenesis of ASA sensitivity and the cross reactions between ASA and indomethacin were discussed. A similar mechanism is likely to be responsible for reactions to NSAIDs and other analgesics. Finally, it should be emphasized that the determination of specific IgE antibodies will hopefully be a diagnostic method for ASA sensitivity.

ACKNOWLEDGEMENTS

This project was supported by a grant of the Forchungsinstitut Borstel, D-2061 Borstel, Germany.

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