

Abnormal platelet hydrogen peroxide metabolism in aspirin hypersensitivity

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Summary

The generation and degradation of hydrogen peroxide (H_2O_2) by platelets was investigated in aspirin hypersensitive and tolerant allergic subjects, and compared to that in general medical patients and healthy controls. H_2O_2 generation following triggering with anti-IgE, specific allergen, and with aspirin (ASA) and indomethacin was examined using a chemiluminescence assay. Platelet levels of the main intracellular enzyme responsible for H_2O_2 metabolism—glutathione peroxidase—were measured by a quantitative functional assay. H_2O_2 secretion could be triggered by anti-IgE in platelets from 15/29 ASA-tolerant atopsics, and by relevant allergen in platelets passively sensitized with allergen-specific IgE. A similar response was observed following triggering with ASA, and with indomethacin, in platelets from 8/25 ASA-sensitive subjects. Mean platelet glutathione peroxidase activity was significantly lower in subjects with ASA-induced asthma, but not in subjects with non-asthmatic reactions to ASA, compared to all other groups. Within the ASA-sensitive group there was no correlation between low peroxidase activity and ASA-triggered chemiluminescence. Our findings emphasize the heterogeneity of ASA-intolerance syndromes and point to abnormal oxygen/peroxide metabolism as a common pathway underlying ASA-induced asthma.

Clinical and Experimental Allergy, Vol. 20, pp. 157–163. Submitted 15 March 1989; revised 11 July 1989; accepted 13 September 1989.

Introduction

Aspirin (acetylsalicylic acid; ASA) hypersensitivity is a common and potentially lethal condition, recognized soon after the drug was introduced in 1899 [1]. It remains an enigma, despite being a potentially highly instructive pharmacological model of allergic inflammation.

The true frequency of ASA-hypersensitivity is difficult to establish. Differences in estimates are related to whether the diagnosis has relied on history or provocation testing, and also in part to real differences in the populations studied, most particularly, whether the primary presentation has been urticaria or asthma. About 4% of asthmatics give a history of ASA-hypersensitivity, but provocation studies indicate higher frequencies with increasing severity of asthma, with some reports suggesting up to 28% when it is associated with nasal polyps [2].

Published series of ASA-hypersensitivity usually show

a preponderance of females and a relatively high age. The condition is often described as having a 'typical' presentation in the third decade. However, it can clearly develop at any age. The rate of development of new cases seems constant throughout life in males, but in females is increased sixfold during the reproductive years [3].

Some epidemiological and experimental confusion could be explained by a failure to distinguish between what may be distinct forms of allergomimetic responses to ASA. Isolated acute urticarial/angioedematous responses without asthma occur, and ASA may also be among the numerous exogenous agents which exacerbate chronic urticaria. The association of asthmatic and naso-ocular reactions to ASA, with recurrent nasal polyps and progressive severe, late-onset asthma, may represent a specific syndrome—'the aspirin triad' [4]. However, ASA-induced attacks can also occur in asthmatics without nasal disease, some of whom are well when not exposed to ASA. Urticaria, and even less frequently colitis, are associated with the respiratory reaction in only a subset of ASA-induced asthma [5].

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The most popular hypotheses concerning the pathogenesis of the condition involve the inhibition of cyclo-oxygenase. Initially an imbalanced effect on bronchoconstrictor and bronchodilator prostaglandins was proposed [6] and subsequently a shunting of precursors from cyclo-oxygenase to lipoxygenase pathways of arachadonic acid metabolism [7]. The main arguments have assumed cyclo-oxygenase inhibition to be the predominant (or only) pharmacological action of ASA, and have depended upon a reported relationship between rates of crossreactivity between NSAID's and their effects on cyclo-oxygenase *in vitro*. However, *in vivo*, cross-sensitivity between cyclo-oxygenase inhibitors is not absolute [6] and no consistent abnormality in prostaglandin or leukotriene production has been demonstrated in tissue from ASA-hypersensitive subjects. *In vivo*, the doses of ASA provoking asthma are sometimes less, and sometimes greater, than those known to completely inhibit platelet cyclo-oxygenase. Also, it is increasingly clear, both that the anti-inflammatory effects of some of these agents are unrelated to cyclo-oxygenase inhibition, and that the group have a number of other potential modes of action [8]. These include other effects on essential fatty acid metabolism [9,10], inhibition of superoxide generation by activated polymorphs [11] and scavenging of hydroxyl radicals [12].

A low avidity receptor for IgE (Fc_ε IIR) has recently been described on platelets, activation through which stimulates a response quite distinct from classical aggregation [13]. It involves a respiratory burst and the secretion of oxygen species detectable by measuring chemiluminescence. Ameisen and Joseph *et al.* [14,15] have described a similar, non-IgE dependent, response in platelets from ASA-hypersensitive asthmatics exposed to ASA *in vitro*. If confirmed, this would clearly have implications both for the mechanisms of ASA-hypersensitivity and for a possible wider role for platelets in asthma, and might also provide a technique for the *in-vitro* clinical diagnosis of ASA sensitivity.

Abnormalities in generation of pro-inflammatory radicals also directs attention to mechanisms that normally limit the biological effects of reactive oxygen species. The most important cytoplasmic enzyme-protecting cells from H₂O₂ is glutathione peroxidase (GPX) [16]. Malmgren *et al.* [17] have reported results which could indicate an abnormality related to peroxide degradation in ASA sensitivity. An increased frequency of lowered peroxidase activity was found in air-dried whole blood samples from 12 food and ASA-sensitive patients. However, that technique is only semi-quantitative, and there are a number of enzymic and non-enzymic peroxidase activities in serum and formed blood elements, including that of haemoglobin. In addition, serum glutathione peroxi-

dase is immunologically quite distinct from the intracellular enzyme [18]. We therefore designed the present study both to evaluate prospectively, platelet chemiluminescence in ASA hypersensitivity and to measure GPX using a specific and quantitative assay.

Subjects and methods

Subjects

Twenty-five ASA-hypersensitive asthmatic subjects were recruited to the study as they presented to a University department of Medicine allergy clinic. Diagnosis was based on a clear history of acute asthmatic or anaphylactic reactions to ASA (with or without similar reactions to other NSAID's), confirmed or refuted by oral provocation studies only where clinically indicated in doubtful cases. Nineteen cases had had acute asthmatic responses to ASA, and six had had acute urticarial/anaphylactic reactions without significant exacerbation of their asthma. Non-asthmatic subjects presenting with chronic urticaria alone were excluded from the study. We also studied 25 normal volunteers and 80 ASA-tolerant atopic subjects presenting to the same clinic. As ASA sensitive and tolerant atopic subjects can be predicted to have a different age and sex spectrum, we also studied 92 unselected subjects presenting to the general medical service of the same department to provide suitable age and sex control data.

Methods

Platelet isolation. Platelet-rich plasma was obtained by centrifuging 10–20 ml of blood anticoagulated with acid citrate dextrose, at $150 \times g$ for 15 min. The platelets were then washed three times in saline buffer containing 36 mM citric acid, 5 mM glucose, 2 mM calcium chloride, 1 mM magnesium chloride, 0.35% serum albumin and 100 nM PGE₁, at 4°C. Platelets were then resuspended in a volume equivalent to half of that of the original plasma with phosphate buffer (PBS), pH 7.6, containing 120 mmol/l sodium chloride and 2.7 mmol/l potassium chloride.

Chemiluminescence. Platelet chemiluminescence was measured using the methods introduced by Prendergast and Proctor [19] and the amplification technique of Whitehead *et al.* [20] as described by Ameisen *et al.* [15]. Briefly, light emission was measured in a Lumac bio-counter (Sonco Ltd, Batley, U.K.) following the addition of triggering reagent to 6×10^5 platelets in 200 μ l PBS containing 720 mU horseradish peroxidase, 418 μ M luminol and 30 μ M luciferin. Counts of photon emission were integrated over 2-sec intervals and recorded every 10

sec. Peak chemiluminescence (as counts per sec) was taken as the maximal value observed within 5 min of the addition of triggering reagent. Four to eight replicates were examined on each occasion and the significance of differences between stimulated and unstimulated emission counts tested using the Mann-Whitney *U*-test. The nature of the chemiluminescence observed was investigated using platelets triggered in the presence of 2 mM catalase (Sigma Chemical Co., Poole, U.K.), 2 mM superoxide dismutase (Sigma Chemical Co.) and heat-inactivated catalase.

Sera containing high titres of allergen-specific IgE were obtained from patient donors and in the *in-vitro* sensitization studies, 3×10^7 platelets were incubated with suitable serum dilutions for 30 min before centrifugation and resuspension in PBS. Polyclonal anti-IgE for triggering was obtained from Miles-Yeda Ltd (Slough, U.K.) and monoclonal anti-IgE's were the gift of Dr P. Wilson. *Dermatophagoides pteronyssinus* and cocksfoot pollen allergens were obtained from Dome Laboratories (Miles Labs, Connecticut, U.S.A.) and the National Institute for Biological Standards and Control (Hampstead, U.K.), respectively. ASA and indomethacin, used in final concentrations up to 0.6 mM and 0.1 mM, respectively, were obtained from Sigma Chemical Co.

Glutathione peroxidase assay. GPX activity was measured in platelet lysates obtained by resuspending fresh centrifuged platelet pellets in saturated digitonin medium [21] at 4°C for 1 hr. Supernatants subsequently stored at -70°C were found to suffer no appreciable loss of enzyme activity.

The coupled assay of Paglia and Valentine [22] was used as modified by Levander *et al.* [23]. Briefly, the volume of lysate was adjusted according to enzyme activity and incubated in a final volume of 1 ml with 2 μ mol glutathione, 1 unit glutathione reductase and 0.3 μ mol NADPH (Sigma Chemical Co.). The reaction was then initiated by the addition of 15 μ l of 15 mM *t*-butyl hydroperoxide and light absorption at 340 nm followed for 4 min. One mU of GPX activity was defined as the oxidation of 1 nmol of NADPH per min and was related to the protein content of the lysate as determined by the Lowry method.

Results

IgE-mediated chemiluminescence

IgE-mediated platelet activation was first investigated using anti-IgE as the triggering agent (Fig. 1). Platelets from 13/29 ASA-tolerant and 4/20 ASA-intolerant subjects gave significant chemiluminescent responses on the addition of anti-IgE. The magnitude of the response to

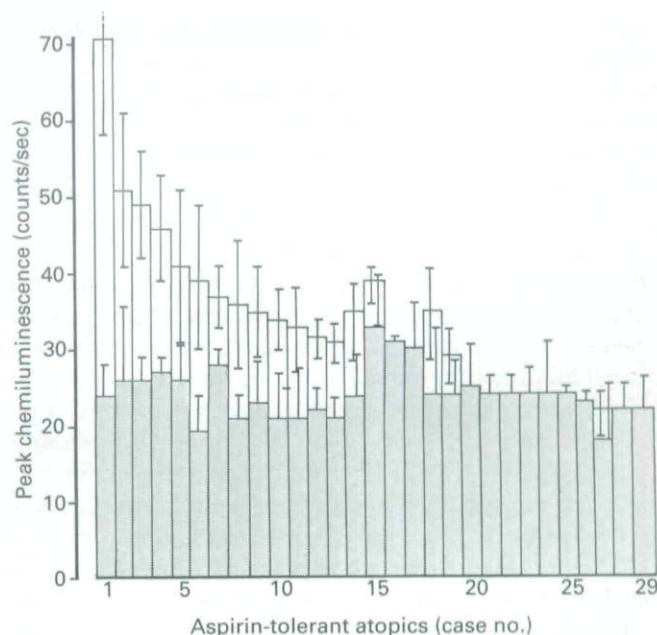


Fig. 1. IgE-mediated platelet chemiluminescence. (□) Platelets from ASA-tolerant subjects triggered with polyclonal anti-IgE following pre-incubation with 110 IU/ml IgE; (■) unstimulated counts. Means and standard deviations of six replicates.

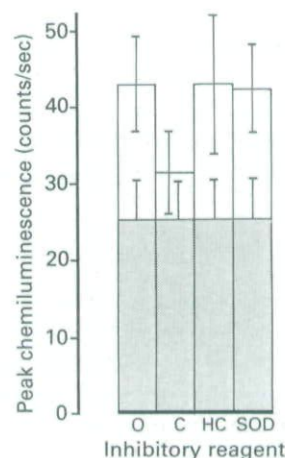


Fig. 2. Inhibition of IgE-mediated chemiluminescence. O, Control; C, 2 mM catalase; HC, heat-inactivated catalase; SOD, 2 mM superoxide dismutase. (□) Platelets triggered with polyclonal anti-IgE without pre-incubation with IgE; (■) unstimulated values. Mean and standard deviation of four replicates from each of four atopic subjects.

anti-IgE was increased in five cases (four ASA-tolerant, one intolerant) by pre-incubation with human serum with a high titre of IgE, and platelets from two further subjects gave positive chemiluminescent responses to anti-IgE triggering after incubation with IgE. At no time in these, nor in subsequent experiments, was platelet chemilumi-

nescence detectable using platelets from non-allergic donors.

Within the atopic population whose platelets were studied on a single occasion, the capacity to react to anti-IgE (with or without pre-incubation with IgE) could not be related to the particular pattern of clinical features expressed by each subject. Nor could the response be related to the type of reaction to ASA in the ASA-intolerant group. However, it was striking that the capacity to produce anti-IgE triggered chemiluminescence varied with time, apparently spontaneously, in a small number of repeatedly retested individuals.

Allergen-specific platelet activation was examined directly without pre-incubation, using suspensions obtained from dust and pollen-allergic subjects. Specific allergen triggering was also investigated after passive sensitization of platelets by pre-incubation with sera containing high titres of specific IgE. The addition of cocksfoot or *D. pteronyssinus* antigens to naturally or passively sensitized platelets produced a burst of light emission similar in magnitude to that elicited with anti-IgE.

Positive luminescent responses to anti-IgE occurred with antibody concentrations which did not produce significant platelet aggregation. The nature of the chemiluminescence response was investigated further in inhibition experiments. Light emission was abolished in the presence of active catalase (Fig. 2), but was unaffected by heat-inactivated catalase, or superoxide dismutase, confirming that the response is dependent upon the secretion of H_2O_2 into the surrounding medium by the platelets.

Platelet activation by ASA and indomethacin

Platelet chemiluminescence in response to the addition of ASA was examined using platelets obtained on one or more occasions from 25 ASA-intolerant subjects. No chemiluminescence was observed after the addition of ASA or indomethacin, at any concentration, to platelets obtained from ASA-tolerant atopic or healthy control donors.

Mean light emission counts following addition of ASA were significantly higher than background and control counts for the ASA-intolerant group as a whole ($P < 0.01$). As can be seen from Fig. 3, platelets from five subjects gave strongly positive responses of a magnitude similar to those produced by maximal anti-IgE and specific allergen triggering. Three further individuals gave smaller, but nevertheless statistically significant, chemiluminescent responses to ASA. Significant increases in light emission could not be detected following the addition of up to 6 mM ASA to platelets from 17 ASA-intolerant subjects.

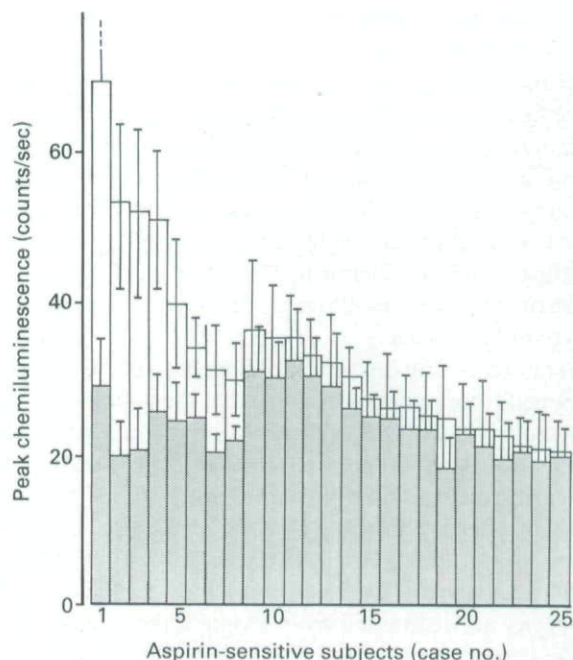


Fig. 3. Platelet chemiluminescent response to aspirin. (□) Platelets triggered with 0.6 mM ASA; (■) unstimulated values. Means and standard deviations of six replicates; cases 1–8, $P < 0.01$.

Table 1. Platelet glutathione peroxidase activity

Group	Numbers males/ females (n)	Mean age (yr) (range)	Platelet GPX (mU/mg protein)	
			Mean	s.d.
ASA-hypersensitive	18/7	45 (23–77)	2.19*	0.17
ASA-tolerant atopics	35/45	34 (16–80)	2.55	0.41
General medicine	43/49	51 (19–87)	2.50	0.39
Healthy volunteers	12/13	34 (20–68)	2.76	0.20

* Significantly different ($P < 0.0001$) from all other groups.

Responding platelets demonstrated a dose-response relationship between ASA-concentration and light emission. Responses to indomethacin mirrored those to ASA. All platelet suspensions reacting to ASA also produced significant chemiluminescence on exposure to indomethacin and none reacted to indomethacin but not to ASA. The capacity of platelets to respond to ASA/indomethacin was consistent on retesting over a period of time in all but one positively responding subject, whose platelets ceased to respond at a time when she was on high dose oral corticosteroids. Failure of ASA/indomethacin to

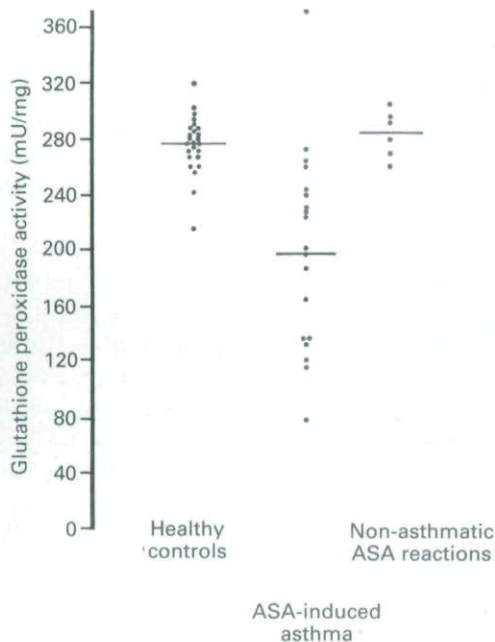


Fig. 4. Platelet glutathione peroxidase activity in ASA-hypersensitive subjects.

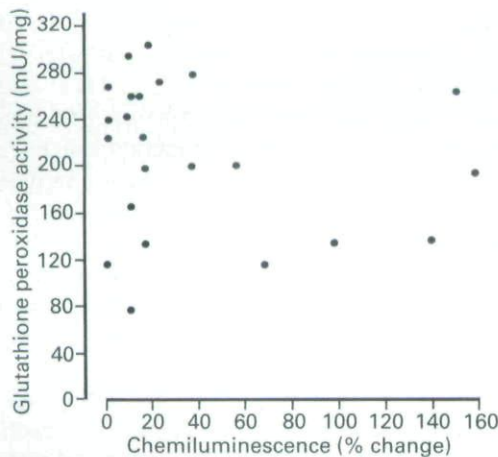


Fig. 5. Platelet glutathione peroxidase activity and aspirin-triggered chemiluminescence.

induce platelet chemiluminescence was also confirmed on multiple repeat tests over a year in one subject with chronic persistent asthma, recurrent nasal polyposis, and provocation-proven intolerance to 10 mg ASA.

Platelet glutathione peroxidase

Platelet GPX activities for each group of subjects are shown in Table 1. No significant influence of age or sex on GPX could be detected. Intergroup differences in GPX activity were highly significant ($P < 0.0001$, ANOVA). Multiple comparison testing (Scheffe) showed that the

mean GPX activity was significantly lower in the ASA-intolerant group than in each of the other groups. Differences in platelet GPX activity between ASA-tolerant atopics, non-atopic medical subjects, and healthy volunteers were not statistically significant.

The range of GPX values for the ASA-intolerant group was greater than that of the other groups of subjects. This appears to be related to the clinical heterogeneity of ASA-intolerance (Fig. 4). Mean platelet GPX activity for subjects with ASA-induced asthma was 1.97 mU/mg protein, compared to 2.84 mU/mg protein for subjects whose urticarial/angioedematous reactions to ASA did not have an asthmatic component ($P < 0.001$). All of the latter subjects had platelet GPX activity close to the mean of healthy volunteers, whereas 15 of the 19 subjects with ASA-induced asthma had GPX levels more than two standard deviations below that mean. Two of these four 'normal' levels occurred in platelets from subjects who were entirely asymptomatic if not exposed to ASA or NSAID's. There was no correlation between lowered GPX activity and ASA-induced chemiluminescence (Fig. 5).

Within the ASA-asthma subgroup, no correlation could be detected between GPX activity and clinical parameters such as rhinitis, nasal polyposis and other associated symptoms. Mean platelet GPX activity was lower in those judged on clinical criteria to have severe asthma than in those with mild disease (1.65 vs. 2.26 mU/mg protein), but this was not statistically significant. However, five of nine classified as severe required maintenance oral corticosteroids which may have elevated GPX activity from lower basal levels. Elevations in GPX activities over those previously recorded were observed in several other subjects during short courses of prednisolone given for acute exacerbations of their disease. Mean platelet GPX activity was similar in those subjects with positive and negative *in-vitro* platelet chemiluminescent responses to ASA.

Within the ASA-tolerant atopic group also, platelet GPX activity could not be correlated with the presence or absence of any particular clinical feature of the syndrome. Fourteen of these subjects had food hypersensitivity. The mean of this subgroup was not statistically different from that for the food tolerant sub-group.

Discussion

The results of our experiments using anti-IgE to trigger platelet chemiluminescence confirms earlier work [14,15] describing IgE-receptors on platelets, that these lead to a form of platelet activation distinct from the classical aggregatory response, and that this includes the generation and release of H_2O_2 . The observation of activation of platelets from allergic individuals with specific allergens

and the passive transfer of this sensitivity with patient serum, supports the view that IgE-mediated platelet activation may be relevant to human reaginic allergy.

Although confirming that similar activation can be triggered by ASA in platelets from ASA-intolerant subjects, our findings do differ from those of the Lille group [15]. They initially reported ASA-stimulated chemiluminescence in platelet suspensions from all of the 14 ASA-sensitive asthmatics studied [15] and a more recent report [24] implies that they have obtained identical results in an expanded study population of 46 subjects. In contrast, we have observed ASA and indomethacin stimulated *in-vitro* platelet chemiluminescence in platelets from only a subset of subjects with ASA-induced asthma and in no subject with non-asthmatic reactions to ASA. Our results indicate that *in-vitro* ASA-platelet activation is not a reliable indicator of *in-vivo* sensitivity. As a diagnostic test it is not very sensitive, but it is highly specific.

Technical factors, including quenching of emitted light by erythrocyte contamination, are unlikely explanations for the above differences: replicate reproducibility was high in our experiments and positive chemiluminescence with anti-IgE could be observed in the same suspensions from ASA-intolerant subjects failing to respond *in vitro* to ASA and indomethacin (and vice versa). Misclassification of our subjects' ASA-status is also unlikely; three asthmatics giving negative *in-vitro* platelet responses to ASA had previously had positive oral provocation tests, however, one of these reactions was so severe that subsequently we considered that double-blind confirmation of sensitivity for experimental purposes could not be ethically justified in subjects giving clear clinical histories of reactions to ASA. Our results cannot be explained by suppression of platelet responsiveness by coincident drug ingestion. We therefore consider it most likely that our findings reflect heterogeneity of ASA-intolerant syndromes.

Abnormal platelet H_2O_2 generation in response to ASA was associated with lowered platelet GPX activity in our ASA-sensitive asthmatics as a group. However, case by case comparisons showed that the two abnormalities are independent. GPX is the most important enzyme degrading H_2O_2 in many cells and tissues [16,25]. Our findings therefore suggest the involvement of H_2O_2 or oxygen radicals as a common pathway in ASA-induced asthma, to which a number of separate abnormalities of oxygen-metabolism regulation could predispose. In addition, reports of IgE-dependent superoxide and/or peroxide production by macrophages [26] and eosinophils [27], as well as platelets, suggest that oxygen radical injury could have a more general role in asthma and allergic disease.

Salicylates and NSAID's are known to have a number

of effects related to oxygen species, most of which would be considered protective rather than pro-inflammatory. Some NSAID's inhibit generation of superoxide by activated polymorphs, probably by blocking ligand-receptor interactions [11]. Although sodium salicylate both fails to inhibit cyclooxygenase and is tolerated by ASA-sensitive subjects, it is considerably more potent than ASA as a free radical scavenger and unlike ASA fails to inhibit the HMPS pathway or interfere with the production of O_2^- and H_2O_2 by granulocytes [12].

The presence of catalase markedly inhibits the generation of factors cytotoxic for *S. mansoni* larvae by platelets stimulated by IgE or by ASA, but the addition of catalase to platelet supernatants fails to abolish cytotoxicity [15], demonstrating the involvement of H_2O_2 in the generation of secondary cytotoxic factors. Similarly, GPX may have a number of direct and indirect effects on the metabolism of several inflammatory mediators. Unlike haem peroxidases which may give rise to free-radical intermediates and multiple oxidation products, GPX yields a single oxidation product, the oxidised form of glutathione (GSSG) [28]. It is capable of reducing a variety of organic peroxidic substrates in addition to H_2O_2 , including hydroperoxides of fatty acids, steroids and prostaglandins [29]. It catalyses the conversion of hydroperoxy products of the lipoxygenase pathway (HPETE's) to the corresponding hydroxy derivatives (HETE's) and may generally downgrade both cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism by removing the trace peroxy-products required for initiation of the reaction [16,29]. In addition, depending on the cause of the lowered GPX observed in ASA-sensitive patients, alterations in the pattern of both lipoxygenase and cyclooxygenase products could result from associated secondary fluctuations in the levels of other enzymes with related activities.

The enzymic activity of GPX is dependent upon the incorporation of a selenocysteine residue into each of its four polypeptide chains [16]. GPX activity is reduced in selenium (Se) deficiency in both animals and man, and platelet GPX activity parallels Se status in experimentally Se-depleted and re-fed animals [18,23,30]. Selenium deficiency adequate to cause lowered GPX activity leads, in some tissues in some species, to a compensatory increase in a non-Se-dependent enzyme with peroxidase activity—glutathione-S-transferase [31]. No information is available concerning human lung in this regard. However, glutathione-S-transferase also catalyses several specific reactions involving the conjugation of both cyclooxygenase and lipoxygenase products, most particularly that generating the sulphidopeptide leukotrienes. We are therefore presently investigating the possibility that abnormal reactivity to ASA could be related to an

alteration in the balance of enzymes with related but different specificities, secondary to nutritional trace element deficiency.

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